STUDIES ON PROTEOLYSIS IN CEREALS

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by

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INTRODUCTION.

The enzymic hydrolysis of the reserve proteins of barley during germination is of major significance in the nitrogen metabolism of the grain. Concomitant with the hydrolysis of the proteins, the proteolytic enzymes of the grain increase in activity manyfold. The products of the proteolysis, the amino acids and the low-molecular weight peptides, rapidly diffuse through the scutellum and are used by the growing embryo partly for the synthesis of new proteins and other nitrogenous substances of metabolic importance and partly, through the respiratory process, as sources of energy (Schuster, 1962). In the brewing process, however, the products of proteolysis formed during the malting and mashing processes ultimately appear in the wort where they provide, at fermentation, non-protein nitrogenous substances for the nutrition of yeast. Certain other products of proteolysis, particularly those of the albumins, endow beer with foam stabilizing compounds, while others contribute to the formation in beer, in conjunction with polyphenols, of various hazes (Preece, 1954; Rainbow, 1960; Djurtoft, 1965). The nitrogen problem in brewing has, therefore, consistently attracted the attention of researchers over a prolonged period.

Despite the importance of the proteolytic enzymes in the nitrogen metabolism of the germinating barley grain as well as of the role these enzymes play in the biochemistry of the malting and brewing processes, few attempts have been made to study, in detail, the proteolytic enzymes of barley and malt. One of the major difficulties has been the lack of availability, until comparatively recently, of substrates which can be employed in reasonably sensitive and reproducible assay procedures. Consequently, little is known about the nature and properties of different types of enzymes which must be present between the initial attack on the native proteins and the ultimate appearance of amino acids (Preece, 1963). Only when the individual enzymes are better understood can the overall transformation of proteins to peptides and amino acids be properly evaluated. Much research, therefore, needs to be done on the nature and specificity of the individual barley and malt proteolytic enzymes, on their natural substrates, on their location in the grain and on their qualitative and quantitative development during the germination of the grain. This thesis reports the results of investigations carried out on some of these topics.

To this date, two general methods have been used to study proteolysis in barley and malt. Either the enzyme(s) have been extracted and studied in vitro using artificial substrates, because of the lack of solubility of the reserve proteins of barley, hordeins and glutelins, in common buffers (Pollock, 1962), or alternatively, ground barley or malt has been autolysed, under standard conditions, in which case the substrate for proteolytic action is provided by the native proteins of barley themselves. In the studies reported here, both these approaches have been employed. In section 1, the autolytic method has been used to study proteolysis in malting barleys, as measured by the release of Water-Soluble, Formol and Non-Protein nitrogen. The object was twofold: to find out varietal differences in proteolysis, if any, and , secondly, its inhibition and activation by certain oxidizing agents and a reducing agent. In section 2, an attempt has been made to fractionate the proteolytic enzymes of malt and to study some of their properties. Section 3 deals with the development of proteases in the germinating barley.

(A) PROTEINS OF BARLEY.

The native proteins of barley are the natural substrates of the proteolytic enzymes of the barley grain. Systematic studies on barley proteins were first reported by Osborne (1895) who classified them into four distinct groups on the basis of their solubilities: albumins, soluble in water and in dilute aqueous salt solutions; globulins, insoluble in water but soluble in dilute salt solution; hordeins, insoluble in water and dilute aqueous salt solutions but soluble in 70% ethanol; and glutelins, insoluble in the above solvents but soluble in dilute acids and alkalis (Preece, 1954; Rainbow, 1960). Later, Bishop (1928; 1929) fractionated the total nitrogen of barley into salt-soluble nitrogen (albumins and globulins) using 5% potassium sulphate, ethanol-soluble nitrogen (hordeins) using hot 70% ethanol, and residual or glutelin nitrogen. The salt-soluble proteins, because of the presence in this fraction of numerous enzymes, have been classed as the cytoplasmic proteins in contrast to the reserve proteins, hordeins and glutelins (Pollock, 1962).

However, more recent studies on barley proteins (see reviews by Brohult and Sandegren, 1954; Danielsson, 1956; Stahmann, 1963) conducted using the newer and more reliable techniques of ion-exchange chromatography, electrophoresis and ultracentrifugation have shown that each of Osborne's groups is a mixture of several components. Whether these components are intact proteins or their degradation products, formed during their extraction using a variety of solvents and subsequent fractionation, has not, however, been clearly established, with the result that considerable differences of opinion exist amongst researchers concerning the composition of barley proteins (see review of literature below). Since methods of extraction of the proteins and fractionation procedures often vary, discrepancies in experimental results reported in the literature are not uncommon. Nevertheless, despite its empirical nature, Osborne's classification has been extensively used as a working basis for subsequent studies on barley proteins.

1. The Water-Soluble Proteins.

The albumin fraction of barley is of considerable significance in the malting and brewing processes as it contains amylolytic and proteolytic enzymes (Enari and Mikola, 1961; Mikola, 1965). Attempts to prepare specific albumins fractions have, however, not met with much success, and as a consequence the true identity of barley albumins has not been established with any certainty. Nevertheless, Danielsson and Sandegren (1947) prepared a specific albumin fraction from barley and reported that the preparation had β- amylase activity which increased with increasing purity of the preparation. A corresponding fraction from malt contained both α- and β- amylase activity although the α-amylase showed properties resembling those of the globulins rather than the albumins (Ayrapaa and Nihlen, 1954). Later, Cooper and Pollock (1957) and more recently Waldschmidt-Leitz (1961) and Reindel and Belohlawek (1961) reported barley and malt albumins to contain several components, each possessing β-amylase activity.

Evidence not related to β- amylase studies has also confirmed the heterogeneity of barley albumins fractionated by ion-exchange and exclusion chromatography. Enari and Mikola (1961) reported that the albumin fraction of barley contained at least sixteen different components, some of which possessed proteolytic activity. Preaux et al. (1962; 1963), on the other hand, fractionated barley albumins into four or five groups of components of decreasing molecular weights. It should, however, be mentioned that in these experiments proteins were characterized by measuring absorbance at 280 mμ. These results should, therefore, be treated with reserve as other compounds, particularly nucleicacids, also absorb at this wavelength (Layne, 1957).

2. The Salt-Soluble Proteins.

The salt-soluble or globulin fraction of barley was first investigated by Quensel using a sedimentation method. Quensel (1942; quoted by Danielsson, 1956) prepared a globulin fraction from barley which separated in the ultraceutrifuge into four sedimenting components viz. α-, β-, γ- and δ-globulins with increasing molecular weights of 26,000, 100,000, 166,000 and 300,000 respectively. Quensel was also able, by ammonium sulphate fractionation, to prepare fractions rich in individual globulins.

Electrophoretic analysis of Quensel's fractions, prepared by Pool and Shooter (1955a) by ammonium sulphate fractionation, however, showed that these were heterogenous and contained two more major components than the four globulins recognised by Quensel. Electrophoretic analysis of saline extracts of barley fractionated with alcohol showed at least six major and eight minor components (Pool and Shooter, 1955b). Djurtoft (1961) classified the salt-soluble proteins of barley into five components according to their sedimentation coefficients in the ultracentrifuge. More recently, Nummi (1963) also fractionated barley globulins, by gel filtration and by electrophoresis into several components.

Of all the components of barley globulins, β-globulin has received the most attention. Because of the presence of β- globulin only in barley and not in any other cereal

(Danielsson, 1949), its survival through malting, unlike the other globulin components (Saverborn et al., 1944) and its high sulphur content, (Sandegren, 1947) like the proteins of chill haze (Jensen, 1952), globulin has been implicated in the formation of chill haze in beer. A recent study (Grabar and Daussant, 1965) has also suggested the implication of the salt-soluble protein of barley in the formation of chill haze in beer.

3. The Alcohol-Soluble Proteins.

Hordeins are the major reserve proteins of barley. Because of their considerable degradation during malting (Bishop, 1929) they provide an important source of supply, during the fermentation process, of non-protein nitrogenous substances for the nutrition of yeast (Preece, 1954). The degradation products of hordeins have been implicated in the formation of haze in beer (Ljungdahl and Sandegren, 1954; Pollock et al., 1959; Grabar and Daussant, 1965), though more recent evidence (see review by Curtis, 1966) suggests that all the proteins of barley play a part in haze formation.

Quensel and Svedberg (1938) analysed a 65% alcohol extract of barley in the ultracentrifuge and found it to be monodisperse with a molecular weight of 27,500. However, Wallis (1951) and Biserte and Scriban (1950; 1952), on the basis of solubility measurements and electrophoretic analysis respectively, showed that hordeins were heterogenous. The latter found that their preparation contained at least five components, viz. α-, β-, γ-, δ-, and ϵ-, hordeins separable by electrophoresis in dilute acetic acid but indistinguishable in the ultracentrifuge. Confirmation of the electrophoretic inhomogeneity of hordeins was reported, later, by Waldschmidt-Leitz and Brutscheck (1955) and by Pollock et al. (1959).

The hordein fractions prepared from barley and malt by Waldschmidt-Leitz and Brutschek (1958) showed high glutamic acid and proline contents. Folkes and Yemm (1956) also showed, by a microbiological assay method, hordeins and glutelins to be far richer than the protoplasmic proteins (albumins and globulins) in amide groups, glutamic acid and proline, these latter amino acids forming over 60% of the nitrogen of the hordeins. On the other hand, the storage proteins were markedly deficient in aspartic acid, alanine, glycine and the basic amino acids lysine and arginine.

4. The Acid - and Alkali - Soluble Proteins.

Glutelins have been the least investigated proteins of barley. They probably include the structural proteins of the grain together with the proteins rendered insoluble because of denaturation during previous extractions with salt and alcohol (Mikola, 1965). Urion and coworkers (Urion and Golovtchenko, 1940; Urion et al. 1944, a, b) reported that about one fourth of the glutelins extracted from barley were in fact albumins and globulins which were not liberated from ordinary relatively coarse barley flours on extraction with salt solutions. If the extractions were, however, made from sufficiently fine flours, the amount of salt-soluble proteins increased from 30 to 40% and the amount of glutelins decreased from 40 to 30%.

Scriban (1951, quoted by Harris, 1962) separated glutelins into α- and β- fractions. Amino acid analysis of the fractions showed no significant differences in composition in spite of the fact that the α- fraction had a nitrogen content of 11.8% and the β- component 15.1% nitrogen. Szilvinyi (1953, quoted by Lundin, 1963), however, reported that glutelins were heterogeneous. He divided them into several fractions according to their solubility in alkali of different concentrations and found that the fractions differed in amino acid composition. Lontie and Voets (1959) divided the glutelin proteins of barley into two fractions, one similar to the hordeins and the other to the salt-soluble proteins, globulins.

(B) PROTEOLYTIC ENZYMES OF BARLEY AND MALT.

1. Classification of Proteolytic Enzymes.

According to Whitaker (1961), Oppenheimer (1923) was among the first to suggest a classification of proteolytic enzymes. He divided them into (a), true proteases, to designate those enzymes which were able to split proteins to peptides but did not attack peptides. Proteases were further sub divided into pepsinases (optimum activity in acid range) and tryptases (optimum activity at pH 6 - 8), and (b) peptidases which were not able to attack proteins but broke down peptides or peptones to amino acids. This classification thus suggested that proteases could be distinguished from peptidases on the basis of the molecular size of the susceptible substrate.

The classification of proteolytic enzymes commonly accepted at the present time is based on the work of Bergmann and coworkers (Bergmann and Fruton, 1941; Fruton et al., 1941; Bergmann, 1942). These workers showed by using synthetic substrates that all proteolytic enzymes attacked peptide bonds and that the difference between proteases and peptidases did not depend on the size of the substrate molecule but rather on the nature of the amino acid side chain and the presence or absence of nearby ionic groups. They divided the proteolytic enzymes into two groups:

(A) the endopeptidases, which were able to hydrolyse the peptide bond provided the amino group and/or the carboxyl group were masked; and (B) the exopeptidases, which were able to hydrolyse the peptide bond provided the amino group and/or the carboxyl group were free. The endopeptidases were further divided into (1), the pepsinases, which required the R^1 (See equation on page 10) group to be either phenylalanine or tyrosine for optimum activity, and preferred that the R group be the same; (2), the trypsinases, which required that R be either arginine or lysine; and (3), the chymotrypsinases, which required that R be either phenylalanine or tyrosine. The plant proteases which resembled papain in their reversible activation and inhibition reactions were included with the endopeptidases. The exopeptidases were divided into (1), the carboxypeptidases, which required that the carboxyl group be free; (2), the aminopeptidases, which required that the amino group be free; and (3), the dipeptidases and tripeptidases, which attacked only di and tripeptides with free terminal amino and carboxyl groups. It was pointed out that with the carboxypeptidases and aminopeptidases size was not a factor as they attacked proteins as well as peptides.

The classification of proteolytic enzymes on the basis of substrate specificity is not completely adequate as a number of enzymes have shown homo-substrate specificity. Trypsin, cathepsin B and the plant enzymes papain,ficin and bromelin, for example, are all able to hydrolyse the synthetic substrate benzoyl- L - arginamide to benzoyl- L - arginine and ammonia.

Proteolytic enzymes have also been classified on the basis of the chemical nature of their active sites (Smith, 1960). On this basis, at present, three different types are recognised. The first group includes enzymes whose activity depends on the presence of one or more thiol groups. These enzymes may be inactivated by heavy metal ions or their derivatives like p-mercuribenzoate and by alkylating agents like iodoacetamide. They are also inhibited by oxidizing agents and, conversely, activated by reducing agents. This group includes papain and other similar plant proteases. A second group includes those enzymes whose activity is dependant on bound metal ions like Mg^{++}, Mn^{++}, Co^{++}, Zn^{++}, Fe^{++} and possibly others. Enzymes of this group are strongly inhibited by metal chelating agents, the commonest used being versene (or ethylenediaminetetraacetic acid). A third category of proteases includes those enzymes which are inhibited by organophosphorus compounds like diisopropylphosphofluoridate. These enzymes are not inhibited by thiol reagents or by metal poisons. Trypsin and chymotrypsin are included in this group.

2. General.

The protein molecule is composed of a large number of amino acids combined through the interaction of the amino group of one of these amino acids with the carboxyl group of a second amino acid to give a peptide linkage (-CO - NH -) as first postulated by Emil Fischer and others (see equation below)

$$R-\underset{NH_3^+}{\overset{H}{C}}-COO^- + H_3\overset{+}{N}-\underset{R^1}{\overset{H}{C}}-\overset{O}{\overset{\parallel}{C}}-O^- \;\underset{\text{synthesis}}{\overset{\text{hydrolysis}}{\rightleftharpoons}}\; R-\underset{NH_3^+}{\overset{H}{C}}-\overset{O}{\overset{\parallel}{C}}-\underset{H}{N}-\underset{R^1}{\overset{H}{C}}-\overset{O}{\overset{\parallel}{C}}-O^- + H_2O$$

Proteolytic enzymes hydrolyse the peptide linkage to yield, ultimately, the amino acids. The susceptibility of the peptide linkage to the (contd. on p. 11)

attack of proteolytic enzymes has been conclusively shown, by the introduction of synthetic substrates of known structures (Bergmann and coworkers; loc. cit.) Studies with synthetic substrates have also shown that in addition to peptide linkages, proteolytic enzymes can also hydrolyse ester, amide and certain other linkages, provided certain specificities of the enzymes are satisfied (see review by Whitaker, 1961).

Except for papain, proteolytic enzymes of plants in general and those of cereals in particular have not been studied in any detail with the result that relatively little is known about the barley or malt proteolytic enzymes. Most of the present day knowledge of proteolytic enzymes has come from studies on proteases of the gastro-intestinal tract of higher animals, which are secreted in relatively high concentrations and are easily freed from other proteins (Green and Neurath, 1954). Barley proteases, on the other hand, have been investigated only in a few cases. Early studies were conducted with methods which were imprecise and often led to conflicting conclusions. Most of the evidence produced from these studies has been rendered inconclusive in the light of more recent findings.

Early studies on proteolytic enzymes of barley and malt have been reviewed by Laufer (1937). According to this reviewer, the existence of proteolytic enzymes in germinating barley was first discovered by Gorup-Besanez (1874). Subsequently, numerous workers attempted to detect the presence of proteolytic enzymes in germinating barley with varying results. In 1900 Fernbach and Hubert, Petit and Labourasse and Windisch and Schellhorn, working independently of each other, established the existence of proteolytic enzymes in barley malt by auto-digestion of aqueous malt extracts, liquefaction of gelatin and by preparation of an active enzyme by extraction with glycerol. Later studies, however, were concerned mostly with the characterization and classification of the proteolytic enzymes of barley malt. Weiss (1903) and later Schjerning (1914), for example, distinguished peptic from tryptic action of malt mainly on the basis of their pH optima. Adler (1915), on the other hand, working with edestin, gelatin and Wittes' peptone as substrates and using the formol titration method for measuring the extent of proteolysis found the existence in barly malt of only one enzyme with optimum activity at pH 4.3-5.0. Lundin (1922) reported the presence of two enzymes in malt: a "peptase" with an optimum pH at 3.1 - 3.4 (gelatin as substrate) and a "tryptase" with an optimum pH at 6.2 - 6.4 (Witte's peptone as substrate).

In the light of classification of proteolytic enzymes put forward by Willstatter and Waldschmidt-Leitz in 1928 (quoted by Hopkins and Burns, 1930), who divided them into proteases and peptidases, several studies were made on malt proteolytic enzymes by Mill and Linderström-Lang (1929), Luers and Malsch (1929), Hopkins (1929), Linderström-Lang and Sato (1929, b) and by Hopkins and Burns (1930). In their main conclusions, these workers agreed that green barley malt contained a protease and a peptidase. The peptidase was separated from the protease by adsorption of all the proteolytic enzymes on an adsorbent, and then selectively eluting the protease at an acid pH and the peptidase at a slightly alkaline pH . The peptidase hydrolysed a synthetic substrate leucylglycine at pH 8.6 and a tripeptide leucylglycylglycine at the same pH . Linderström-Lang and Sato (1929 b) reported that green malt also contained an enzyme which hydrolysed another synthetic dipeptide alanylglycine at an optimum pH of 7.6. The optimum pH for the action of malt protease also varied with the substrate employed, being 4.9 - 5.0 for gelatin, 4.3 for edestin, 4.6 for egg albumin and Witte's peptone and 3.3 - 5.5. for casein. It should, however, be pointed out that the use of synthetic di- or tri-peptides as substrates in the early studies did not necessarily confirm the presence of di- or tri-peptidases in the malt. These substrates were non-specific and according to present understanding of proteolytic enzymes (see classification of proteolytic enzymes on page 8) were probably hydrolysed by proteases as well as by peptidases present in the malt.

More recent work has also been directed towards gaining an understanding of the proteolytic enzymes of barley and malt. Kringstad and Kilhovd (1957) divided them into three fractions according to their solubility: Water-Soluble, Salt-Soluble (1M NaCl) and those which were neither water nor salt-soluble. The three fractions showed different development during malting, exhibited different properties towards various enzyme activators and showed different thermostability at 70°C., the insoluble fraction being the most stable. More recently, Enari et al. (1963) showed barley and malt to contain five different water-soluble proteases including one which hydrolysed a synthetic substrate α - benzoyl - arginine - p - nitroanilide. The presence of this enzyme in barley extracts was confirmed later by Suolinna et al. (1965) and by Burger (1966).

3. Development During Germination.

It was reported as early as 1900 by Windisch and Schellhorn (quoted by Pollock, 1962) and later by others (Bloch, 1939;

Rainbow, 1960) that ungerminated barley contained little proteolytic activity. The proteolytic activity of the grain, however, increases rapidly during germination, especially after four or five days of growth (Hopkins, 1929, 1943; Enari et al., 1963), the proteases appearing to precede the peptidases, according to Waldschmidt-Leitz and Purr (1931). Concomitant with the increase of proteolytic activity, there is a simultaneous diminution in the reserve proteins, the hordeins and glutelins (Rainbow, 1960).

However, the development of proteolytic enzymes in germinating grain, following steeping, depends on the condition of the grain as well as on the malting conditions. The proteolytic activity of whole wheat seed was shown by Mounfield (1936a and b, 1938) to increase about tenfold during germination. The extent of increase was approximately halved when the seeds were stored for 14 to 16 months prior to germination. On the other hand, maximum activity was obtained when wheat was stored for two months prior to malting.

The germination of barley at low temperature leads to greater enzyme production, the optimum temperature being 13 - 17°C (Harris, 1962). Kolbach and Schild (1939), for example, showed that the formol nitrogen in extracts of malt decreased as the temperature of germination was raised from 13 - 25°C.

Urion (1950) reported that the maximum content of soluble nitrogen in germinating barley was reached after five days, after which there was little change in this respect. Kringstad and Olsen (1955) reported that the total proteolytic activity of barley increased up to a maximum in nine days; thereafter there was a decrease in activity lasting five to seven days followed by an increase up to 22 days. In a later report on the development of water- soluble, salt-soluble and insoluble proteases, Kringstad and Kilhovd (1957) showed that the insoluble proteases attained maximum activity on the third day of germination, whilst the other required four to five days to reach this stage thus indicating a qualitative difference in the development of proteases during the germination of barley.

4. Distribution in the Grain.

The distribution of proteases in barley has been studied only in a few cases, probably because of the difficulty of separating the grain into reasonably pure individual fractions. There are, in general, three distinct regions of the barley grain which show, during germination, microscopical evidence of the presence of enzymes including proteases (Schade, 1937). These regions are (a), the scutellar epithelium layer (the layer which forms the boundary between the scutellum and the endosperm) (b), the aleurone layer and (c), the inner endosperm, the latter term being used to designate the endosperm minus the aleurone layer (see Harris, 1962).

During germination of the barley grain, the scutellar epithelium layer secretes a considerable quantity of the proteolytic enzymes into the endosperm, which contains the storage proteins. Dickson and Burkhart (1942) reported that 70 - 80% of the total proteolysis in malting and brewing occurs during germination in the half of the grain containing the embryo. Confirmation of the presence of a protease (tested on edestin) and a dipeptidase (tested on dl-alanylglycine) in the scutellum and none or little in the hull and endosperm of the dormant and germinating wheat seed was reported by Pett (1935). Pett, however, did not separate the aleurone layer of the grain which contains considerable quantities of the proteolytic enzymes (Engel and Heins, 1947).

While the ability of the scutellum to secrete proteolytic as well as other hydrolytic enzymes was generally conceded, the enzyme-secreting ability of the aleurone layer was vigorously disputed by the early workers. Stoward (1911, quoted by Schade, 1937), however, produced strong evidence which suggested that the aleurone layer has enzymic potential comparable to that of the scutellum. Stoward showed that when the aleurone cells were left in contact with the endosperm in the absence of embryos, dissolution of the endosperm proteins and other reserve materials occurred which suggested that the aleurone secreted the enzymes responsible for the dissolution of these materials. On the other hand, when the endosperm alone was put under conditions of germination, little or no depletion of the reserve materials occurred. Later Gruss (1928) confirmed Stoward's findings by investigating the various enzymes produced by the aleurone layer.

Engel and Heins (1947) studied the distribution of proteases in three cereals fractionated using a histological technique and assayed the enzymes using substrates previously employed by Pett (loc. cit.) . They reported that the embryo and the aleurone cells of wheat, rye and oats were rich in a proteinase and a dipeptidase. The endosperm, on the other hand, contained only very small or a negligible amount of these enzymes. Whether the same pattern of distribution is maintained during the germination of the grain is, however, not known as Engel and Heins investigated the distribution of proteases only in the dormant grain.

The foregoing brief evidence, therefore, suggests that in barley, presumably like in other cereals, almost all of the proteolytic enzymes are present in two regions of the grain viz. the aleurone and the scutellar epithelium layers.

5. Solubility.

The extraction of proteolytic enzymes from barley grain presents a major difficulty. Not only are the enzymes present in extremely small quantities, but they are also not soluble in most of the common enzyme extractants. It has been recently shown (Kringstad and Kilhovd, 1957) that only part of the proteolytic enzymes of barley and malt are extractable by water, part being extractable by dilute aqueous salt solutions and a further part being insoluble in water or in salt solutions. Recently, McDonald and Chen (1964) also reported the presence of insoluble proteases in wheat flours (these proteases were not extractable in an active form by acetate buffers (0.1 - 0.4 M, pH 4.6 or 0.1M, pH 3.8); 0.2 M-NaCl, pH 7.5; borate-acetate buffers, pH 8.2; or in 10% sodium chloride, pH 5.8).

Insoluble proteases thus constitute an important fraction of the total proteolytic activity of barley and malt. In procedures, where water or salt solutions have been employed as extracting agents, obviously, not all of the proteolytic activity is extracted.

6. Activation and Inhibition.

Although few studies have been reported, with purified preparations, nevertheless, it is generally believed that the proteolytic enzymes of barley and malt, like those of wheat isolated by Hall and Hale (1936, 1938) and Hale (1939) are of the papain type and, therefore, need thiol groups for activity. These enzymes are activated by reducing agents like cysteine, glutathione and thioglycollic acid (Waldschmidt-Leitz and Purr, 1931; Kringstad and Kilhovd, 1957) and inhibited by oxidising agents (Macey and Stowell, 1961). Specific inhibition of the thiol enzymes is also achieved by using mercury derivatives and by alkylating agents (Smith, 1960).

Recent studies conducted with wheat by McDonald and Chen (1964) and with barley by Enari et al. (1963) have shown that papain-type enzymes in these cereals may account for only part of the total proteolytic activity. Enari et al. reported the presence of an enzyme in water extracts of barley and malt which was inhibited by an unidentified low-molecular weight inhibition present in the water extracts of the grain, though not by potassium bromate, iodate or ethylenediaminetetra acetic acid or by sulphydryl enzyme inhibitors as reported, later, by Burger (1966). Removal of the low-molecular weight inhibitor from the grain extract by gel filtration on Sephadex G - 25 caused a fourfold increase in the activity of this enzyme. Enari et al. (1964) also found proteolysis during mashing to be inhibited by proteins and smaller molecules present in barley extracts. In a recent paper, however, Suolinna et al. (1965) reported a partial (50 - 70%) inhibition of this enzyme by specific SH poisons and suggested that the thiol group did not seem to be at the active centre of the enzyme but sufficiently close to it to interfere sterically with the formation of the enzyme-substrate complex.

The nature of the activation process of the SH enzymes has been the subject of controversey. The bulk of the evidence obtained from studies on papain favours the theory that the activator regenerates the thiol group if the enzyme is oxidized. The activation is reversed by oxidizing agents. The inhibited enzyme can be reactivated provided the thiol group has been only oxidised and not alkylated (Green and Neurath, 1954).

7. Assay Methods.

The proteolytic enzymes of barley or malt have been assayed either by using an autolytic method as used by Hopkins and Kelly (1931), Idoux (1933 quoted by Harris, 1962); and more recently by Preece (1963), or by using an extract method in which case isolated enzyme preparations have been assayed using artificial substrates like gelatin, haemoglobin, casein and human serum albumin (Massart, 1946; Sandegren and Klang, 1950; Enari and Mikola, 1961; Enari et al., 1963; Grabar and Daussant, 1963). The use of artificial substrates, though not ideal, is however, unavoidable in view of the low activities obtained with the autolytic method (Zoch and Olson, 1949; McDonald and Chen, 1964) and its unsuitability in the purification and characterization of the proteolytic enzymes of barley or malt. Most of the information on barley proteases has, therefore, come from the use of substrates from sources other than barley.

The assay methods which employ artificial substrates my be classified as those which either measure the disappearance of the protein substrate or the appearance of hydrolytic products. The methods which measure the appearance of hydrolytic products, liberated from various substrates, include the Van Slyke procedure (1911); titration with acid in acetone (Linderström-Lang, 1928); increase in formol nitrogen as used by Bishop (1943) and by others and the haemoglobin assay method of Anson (1938) used with modification by Sandegren and Klang (1950). Carboxyl groups liberated have been measured by titration in ethanol (Willstatter and Waldschmidt-Leitz, 1921). Alternatively, the disappearance of protein substrate as measured by the decrease in viscosity of gelatin solution has been used by Koch and coworkers (1939, 1942); Massart (1946), and more recently by Enari et al. (1963), as measure of proteolytic activity of barley or malt. The methods most commonly employed have been, however, the viscosity reduction method, the formol titration method and the haemoglobin assay method of Anson.

The viscosity reduction method suffers from the disadvantage that if the protein molecule is split close to the centre a marked decrease in viscosity would occur. On the other hand, a split near the ends of the protein molecule would not appreciably change the viscosity (Whitaker, 1961). Nevertheless, the viscosity reduction method can be used with advantage to distinguish between endo- and exopeptidases.

The formol titration method employs the principle of blocking the amino groups with formaldehyde to form derivatives which are more acidic than the original amino compounds and are titrated with alkali to derive a measure of the amino groups present in the material assayed. The reaction of α- amino groups with formaldehyde was originally explained by Sørensen (1907) as shown in the following equations:

$$^{-}OOCRCHNH_3^{+} \rightleftharpoons {}^{-}OOCRCHNH_2 + H^{+}$$

$$^{-}OOCRCHNH_2 + HCHO \rightleftharpoons {}^{-}OOCRCHNHCH_2OH$$

$$^{-}OOCRCHNH_2 + 2HCHO \rightleftharpoons {}^{-}OOCRCHN(CH_2OH)_2$$

In formol titrations the protein or amino acid solution is first titrated with alkali (sodium hydroxide) to a given pH (this titration being referred to as preliminary titration), then formaldehyde of a neutral pH is added and the resulting solution is titrated with alkali to an end point at the same or a different pH. The second titration is usually taken as a measure of the release of amino nitrogen. The precise pH to which the preliminary titration should be taken, however, varies. Values ranging from pH 6 to 9 have been used by a number of workers (see Taylor, 1957) in formol titrations of pure peptides and amino acid solutions. The pH values used for preliminary titrations in other studies may not, however, be suitable in formol titrations of autolysates of barley, which contain many other alkali - titrable groups in addition to the amino groups liberated by the enzyme (s) action during the autolysis of the grain. It may, therefore, be necessary to determine, under these conditions, a pH suitable for preliminary titration.

The haemoglobin assay method, originally developed by Anson, has gained the widest acceptance for the assay of proteolytic enzymes and older methods have largely been superseded by this sensitive procedure. This method was, therefore, chosen as an assay procedure for the determination of the total proteolytic activity of the grain. In this method, the substrate solution is prepared from urea-denatured haemoglobin (other proteins may also be used). After the enzyme has been allowed to act on the protein for a fixed time, trichloroacetic acid (TCA) is added to terminate the reaction and the precipitate undigested protein. After filtration or centrifugation the tyrosine and tryptophan - containing peptides are estimated either by using the color given with the Folin-Ciocalteau reagent or by measuring extinction at 280 mu (Davis and Smith, 1961). Recently, McDonald and Chen (1965) have suggested a modification which measures total peptides present in the TCA-filtrate and permits estimation of very low levels of protease activity.

More recently, synthetic substrates have been employed to characterize the proteolytic enzymes of barley and malt (Enari et al., 1963; Suolinna et al., 1965; Burger, 1966). The availability of synthetic substrates offers great promise in future studies on the proteolytic enzymes of barley, particularly on their specificity as has been done with the proteases from the gastro-intestinal tract of higher animals and microbiological sources.

The choice of an assay method will, however, depend on the purpose of an investigation. While in vitro investigations with purified enzyme preparations are necessary to study and characterise individual barley and malt proteases as has been done recently (Enari et al., 1963; Suolinna et al., 1965; Burger, 1966), such studies may be of limited value in malting and brewing as the substrates employed are not those which the enzymes encounter during the malting and mashing processes. Perhaps in this respect the autolytic method, followed by the determination of non-protein nitrogen, offers an advantage. It provides some indication of the course of events likely to occur in barley on steeping. Furthermore, in the autolytic method, proteolysis proceeds in conjunction with other enzymic changes taking place in the grain.

8. <u>Fractionation Procedures.</u>

Proteolytic enzymes of barley and malt, like other enzymes, may be fractionated by using most of the procedures available for the fractionation of proteins. Although recently a number of attempts have been made to fractionate plant proteolytic enzymes (Soedigdo and Gruber, 1960; Kramer and Whitaker, 1964; Scarbieri et al., 1964), the fractionation of the proteolytic enzymes of barley or other cereals using modern methods of enzyme purification has been attempted only in a few cases. However, at present, a large number of methods are available for the fractionation of proteins (see review by Sober et al., 1965) and any of these methods may be used for the fractionation and purification of barley or malt proteases. It is not intended here to review all the available methods (they have been discussed in detail by Sober et al., loc.cit.) but only to describe very briefly those procedures which are more commonly used, at present, in enzyme prification. Most of these methods have been employed recently by Enari et al. (1963) and by Burger (1966) for the fractionation and purification of barley proteases. These procedures may be listed as:

1. <u>Ammonium Sulphate Fractionation.</u>

Fractionation with salt is the classical method of enzyme purification (Dixon and Webb, 1959); the salt most commonly used being ammonium sulphate. Since different proteins are "salted out" at different salt concentrations, by varying the ionic strength of the salt an enzyme mixture may be precipitated into different fractions.

2. Fractionation with Organic Solvents.

The systematic purification of proteins by means of organic solvents was introduced by Cohn and Coworkers (1946). Organic solvents, by virtue of their low di-electric constant, reduce the di-electric constant of aqueous protein solutions and thus bring about their precipitation. Again by varying the concentration of the organic solvent, an enzyme mixture may be resolved into different fractions.

3. Chromatography.

a. Adsorption.

Basically this procedure consists of adsorption of enzyme protein to an inert material (like calcium phosphate gel) followed by selective elution of the enzymes from the adsorbent. Alternatively, if the enzyme is not adsorbed, treatment with the adsorbent may be used to remove the unwanted materials from the enzyme solution. Adsorption chromatography was employed in some of the early studies (Luers and Malsch, 1929; Hopkins, 1929; Linderstrom-Lang and Sato 1929a) to separate peptidases from proteases present in 'green' malt.

b. Ion-Exhhange.

The enzymes, being proteins, will usually exist in solution either as cations or anions and as a result will bind to the ion-exchange materials like carboxymethyl (CM) - cellulose and diethylaminoethyl (DEAE) - cellulose depending upon the pH of the solution, since pH affects the ionization of the exchange material as well as of the proteins. The adsorbed proteins may be resolved into separate fractions either by raising the salt concentration (salts reduce the electrostatic attraction between the ion-exchange material and the proteins) or by changing the pH by means of buffer solutions.

c. Exclusion Chromatography or Gel Filtration.

Exclusion chromatography has been widely used for the separation of enzymes since Porath and Flodin (1959) used it for the first time for desalting an aqueous solution of proteins. Since separation of a mixture by this method is achieved exclusively on the basis of molecular size, only enzymes having widely varying molecular sizes are likely to be separated.

4. Electrophoresis.

The electrophoretic separation of proteins is based on the differential migration of ions in an electric field, the rate of migration (and charge on the protein) being dependant on the pH of the medium. Electrophoresis thus operates on a different principle to chromatography and, therefore, offers an alternative procedure for the fractionation of enzymes.

In any scheme of enzyme purification, the ultimate objective is to separate the enzyme (s) into a single peak of activity which is relatively free from other proteins. This is, however, rarely, if ever, possible by using only a single method of purification. Therefore, a combination of methods need to be employed, though even then there is no guarantee of success.

In these studies (section 2), the methods used for the fractionation of proteases from malt were the ammonium sulphate precipitation and exclusion and ion-exchange chromatography. These methods were employed on the assumption that the malt contained proteases which were of different molecular weight, and possessed such other characteristics by which they were liable to be fractionated by these methods.

EXPERIMENTAL.

(A) SECTION 1: AUTOLYSIS OF BARLEY AND MALT.

1. MATERIALS.

(a) Grain.

To study proteolysis in malting barleys, six barley varieties, viz., Hunter, Maythorpe, Mentor, Proctor, Swallow and Ymer, all of the 1963 harvest, were used. The moisture contents of the varieties, determined according to the recommended method of the Institute of Brewing (1961) were 13.2, 14.0, 13.2, 13.0, 13.7 and 13.0% respectively. When the effect of inhibitors and an activator on proteolysis was studied, Ymer barley and Proctor malt (distiller's malt kilned at 125 - 140°F; moisture content 8.6%) made from Proctor barley of the 1963 harvest, were used. The malt was kindly supplied by Mr. T. Fergusson of Archibald Campbell, Hope and King Limited, Edinburgh.

(b) Reagents.

All chemicals used were of AnalaR or of laboratory reagent grade. Potassium bromate, phenylmercuryacetate, iodoacetic acid and thioglycollic acid (Na salt) were obtained from British Drug Houses Limited.

(c) Apparatus.

The pH of the solutions was measured, throughout the experiments, using the Pye "Dynacap" pH meter. The autolysates were centrifuged using a Gallenkamp (Universal) centrifuge.

2. METHODS.

(a) Preparation of Grain Autolysates.

Autolysates of barley or malt were prepared according to a method described by Preece and Aitken (1953). The equivalent of 25 g. dry weight of barley or malt, finely ground by hand in a coffee mill, was mixed in 250 ml. centrifuge bottles with 100 ml. of distilled water or, when the effect of inhibitors or an activator or proteolysis was studied, with a solution of appropriate concentrations of potassium bromate, phenylmercuryacetate, iodoacetic acid or thioglycollic acid (Na salt) previously heated to 40°C. The inhibitors and activator solutions were adjusted with 1N hydrochloric acid or sodium hydroxide to pH 6.0, which was the pH of the extract when the grain was autolysed with distilled water (used as control). The mixture was well shaken to ensure complete wetting of the grain and autolysed in a constant temperature water bath at 40°C, shaking every 15 minutes, for t - 11 minutes where t was 15, 30, 60, 120 and 240 minutes and 11 minutes the time required to centrifuge the mixture. After appropriate times, the bottles were removed from the water bath and centrifuged at 2500 r.p.m. to remove the insoluble materials and the supernatant fractions filtered through glass wool to remove the hull fragments. Suitable aliquots of the bright filtrate were taken to determine, in duplicate, Water-Soluble nitrogen, Formol nitrogen and Non-Protein nitrogen, which are expressed as mg. per 100 g. dry barley or malt.

(b) Water-Soluble Nitrogen.

Water-Soluble Nitrogen in the filtrate was determined by the micro-Kjeldahl method. 5 ml. of the filtrate were mixed with ca. 0.5 g. of a catalyst mixture composed of potassium sulphate, copper sulphate pentahydrate and selenium in the proportions 100: 6: 1 (Institute of Brewing, 1961) in a 50 ml. micro-Kjeldahl flask and digested until clear (usually in 30 - 40 minutes) with 5 ml. of concentrated sulphuric acid. The digest was cooled, diluted with distilled water and the flask connected to a distillation assembly. Sodium hydroxide solution (40%, approximately 10 ml., boiled to remove traces of ammonia and cooled) was added and the ammonia liberated distilled into 2% (w/v) boric acid solution containing 3 drops of a mixture of methyl red and methylene blue indicator (A.O.A.C., 1960), and titrated with standard hydrochloric acid (0.05N). The accuracy of the procedure was checked with a solution of glycine (99% purity) of known nitrogen content and was found close to 99% as shown below in Table I.

Table I - Determination of efficiency of the micro-Kjeldahl procedure for the estimation of nitrogen.

No.	% nitrogen in glycine solution.	% nitrogen estimated by the micro-Kjeldahl procedure.	% recovery.
1	0.070	0.068	97.1
2	0.070	0.070	100.0
3	0.070	0.069	98.5
		Mean	98.5 ± 0.84 (SEM)

(c) Formol Nitrogen.

Preliminary experiments were first conducted to determine a pH (hereafter called the pH of inflection at which probably most of the titrable acidity of the autolysate, due mainly to the presence of carboxyl groups, was neutralized) to which the grain autolysates were later titrated before the addition of neutralized formaldehyde. A number of barley varieties, both of the 1962 and 1963 harvest, were used in the preliminary titrations. The grain was autolysed with distilled water as described earlier and an aliquot (50 ml.) of the bright filtered extract was titrated potentiometrically usually to pH 10 - 11, while being continuously stirred mechanically, by adding from the burette small portions of the standard sodium hydroxide (0.05N). The pH of the solution was measured after each addition of the sodium hydroxide and the data plotted graphically (pH vs volume of 0.05 N NaOH) to obtain a titration curve. Fig. 1 shows, for the purpose of illustration, a typical titration curve of a 240 minute autolysate of Proctor barley of the 1963 harvest. Similarly, titration curves were obtained for autolysates prepared from different barleys, and for autolysates prepared from the same barley autolysed for various periods of time. The results of preliminary titrations showed that, in each case, the slopes of the titration curves were more or less similar: an initial substantial linear increase in the pH after which an inflection occured followed by a linear phase again. If a tangent was drawn to the upper linear portion of the curve parallel to the lower linear portion of the curve, which was extrapolated to obtain a straight line, a line could be drawn (the middle line, see Fig. 1) at an equidistance to the upper linear and the lower linear portions of the curve. The point at which the middle line intersected the titration curve was taken as the point of inflection of the titration curve. From this point the pH of inflection of the curve and the corresponding titre (ml. of 0.05 N NaOH) for that pH was determined as in the case of the data plotted in Fig. 1 . Similarly, the pH of inflection and the corresponding titre was determined for titration curves of autolysates prepared from different barleys and from the same barley autolysed for different periods of time. Some selected results of these preliminary titrations are given in Tables ii, iii and iv. These results show that under the conditions used in these experiments, the mean pH of inflection estimated was 8.5 ±. On the basis of these results, all subsequent formol titrations were conducted in the following manner. An aliquot (50 ml.) of the autolysate, obtained from barley or malt, was titrated potentiometrically to pH 8.5 with 0.05 N sodium hydroxide and the titre noted (titre a); this titre being taken as the acidity contributed mainly by the carboxyl and other acidic groups present in the autolysate. Then excess (60 ml.) of formaldehyde previously neutralized to pH 6.8 by the addition of unstandarized sodium hydroxide was added and thoroughly mixed in. The addition of formaldehyde caused a fall in the pH of the mixture. After five minutes standing at room temperature, the mixture was titrated to pH 8.5 with 0.05 N sodium hydroxide (titre b); this titre being taken as a measure of the release of amino nitrogen liberated by proteolysis.

It should, however, be pointed out that it is not easy to suggest with any certainty as to what exactly is being measured in these titrations by titre a or by titre b. Titre b which has been taken in these titrations as a measure of the release of amino nitrogen liberated by proteolysis may give a lower estimate of the amino nitrogen as according to Mounfield (1936 a) some of the amino nitrogen is partly titrated in the preliminary titration (titre a). Mounfield, therefore, used the sum of titres a + b as an index of proteolytic activity of the wheat grain. This index, in Mounfield's view, is a more reliable estimate of the total hydrolysis of the peptide linkage. The results of formol titrations can, therefore, be only relative and were obtained in conjunction with the measurement of the release of the Water-Soluble and Non-Protein nitrogen (Lundin's fraction C); the latter nitrogen fraction according to Lundin (1953) gives an estimate of formol nitrogen which is more accurate and independent of the subjective influences in formol titrations.

(d) Non-Protein Nitrogen.

Non-Protein nitrogen in the filtrate was determined by the method of Lundin (1953) in which phosphomolybdic acid is used as a protein precipitant. In this method the nitrogen which is not precipitated by phosphomolybdic acid represents mainly the amino and low-molecular weight peptide nitrogen (Lundin's fractionC). 25 ml. of the filtrate were precipitated, in a conical flask, by adding 2 ml. each of 50% (v/v) sulphuric acid and of 50% (w/v) sodium molybdate. The flask was held at room temperature (18 - 20°C) for 10 minutes to ensure a thorough precipitation of the proteins, after which the contents were filtered through Whatman No. 1 filter paper. An aliquot of the filtrate was taken to determine total nitrogen by the micro-Kjeldahl method as described above.

(e) Total Nitrogen.

Total nitrogen content of barley or malt was determined by the macro-Kjeldahl method. This method is described in detail in the recommended methods of analysis of the Institute of Brewing (1961).

(B) SECTION 2: FRACTIONATION OF PROTEOLYTIC ENZYMES OF MALT.

1. MATERIALS.

(a) Grain.

Barley malt previously described on page 27 was used for the extraction of proteolytic enzymes. To reduce deterioration, the malt was stored at 4°C.

(b) Reagents.

All reagents used were of AnalaR or of laboratory reagent grade. N - α - benzoyl - DL - arginine - p - nitroanilide (BAPA) hydrochloride, N - α - benzoyl - L - arginine ethyl ester (BAEE) hydrochloride, benzoyl - L - arginamide (BAA) hydrochloride monohydrate, N - acetyl - L - tyrosine ethyl ester (ATEE) monohydrate, and bovine serum albumin (crystalline) were purchased from British Drug Houses Ltd. Commercial bovine haemoglobin, scales, (Hb) was always obtained from the same source (Koch-Light Laboratories Ltd.) as Kringstad and Kilhovd (1957) had reported poor reproducibility of results when commercial haemoglobin obtained from different sources was used in enzyme assays. The specific SH enzyme inhibitors p - hydroxy-mercuribenzoate (Na salt), p - chloromercuriphenyl - sulfonic acid, iodoacetamide and N - ethylmaleimide and a specific trypsin inhibitor phenylmethylsulphonylfluoride (Fahrney and Gold, 1963) were supplied by Sigma (London) Chemical Company Limited. Sephadex G-75 and G-100 were obtained from Pharmacia (G.B.) Limited. The anion-exchanger diethylaminoethylcellulose (Whatman powder DE 50) was purchased from British Drug Houses Limited.

(c) Apparatus.

All optical extinctions were measured in a Unicam SP 500 Spectrophotometer using glass or silica cuvettes of 1 cm. light path. The grain extracts were centrifuged using an MSE Medium or MSE 18 refrigerated centrifuge at $0^{o}C$. The proteins precipitated with ammonium sulphate were recovered using an MSE 18 refrigerated centrifuge at $0^{o}C$. In column chromatography fractions were collected using an automatic fraction collector. For lyophilization of the extracts, an Edwards High Vacuum freeze-dryer was used.

2. METHODS.

(a) Extraction of Enzymes.

(i) Extracting Agent.

To this date, a number of solvents have been employed by various workers for the extraction of proteolytic enzymes from the cereal grain. In view of the low solubility of these enzymes in most of the common enzyme extractants (Kringstad and Kilhovd, 1957; McDonald and Chen, 1964) and the lack of availability of a generally acceptable extraction procedure, a number of solvents were first employed for the extraction of proteolytic enzymes from the malt. The solvents used were (a), distilled water as used by Enari et al. (1963) (b), 0.15M sodium chloride solution as recommended by Morton (1955) and (c), 5% (w/v) potassium sulphate as used by Macey and Stowell (1957); this latter salt is also employed at this concentration for the extraction of the salt-soluble proteins (albumins and globulins) of barley (Preece, 1954).

10 g. of the malt, hand-ground in a coffee mill, were extracted, in each case, with usually 3 volumes of the extracting

agent for one hour by shaking mechanically at room temperature (18 - 20°C). The debris was removed by straining the extracts through two layers of cheesecloth and the extracts were centrifuged at 10,000 x g. The supernatant fractions containing a few drops of toluene as a disinfectant were dialysed over-night, at room temperature, in Visking dialysis tubing (32/32") against running tap water. The non-diffusible materials were recentrifuged and the supernantant fractions made to volume (50 ml.) with distilled water. One ml. aliquots, from each fraction, were taken to determine proteolytic activity (Hb-ase) using haemoglobin as a substrate according to a procedure described on pages 38-40.

The results show (Table v) that a more active enzyme preparation was obtained when the malt was extracted with 0.15 M sodium chloride solution or with 5% potassium sulphate than when it was extracted with distilled water. Although the 5% potassium sulphate extract showed, in most cases, a higher Hb-ase activity than the extract obtained with 0.15M sodium chloride (Table v), subsequently all the enzyme extractions were carried out with 0.15 M sodium chloride solution because of a low assay blank obtained with this extracting agent. Potassium sulphate (5%) apparently extracted more salt-soluble nitrogen which contributed to an exceptionally high assay blank.

In another set of experiments, the effect of L-cysteine - hydrochloride on the Hb-ase activity of malt was studied. 10 g. of the ground malt were extracted, as described above, with 0.15 M sodium chloride solution containing 0.5% (w/v) L- cysteine - hydrochloride (British Drug Houses) neutralized to pH 7.0 with sodium hydroxide. The results of Hb-ase activity measurements presented in Table (vi) indicate that the addition of 0.5% L - cysteine - hydrochloride to the extracting agent resulted in a slight increase in the Hb-ase activity of the malt.

(ii) Treatment of the Grain.

Kringstad and Kilhovd (1957) had reported that a considerable portion (about one-third) of the proteolytic enzymes of malt were not extractable by water or 1M sodium chloride solution. It is possible that the insoluble proteases in malt may be present as lipo-protein complexes. Various organic solvents (see Morton, 1955) are used to dissociate the cell lipo-protein complexes so as to facilitate the release of enzymes. In a preliminary experiment, the grain was treated with acetone, prior to enzyme extraction, as described below.

25 g. of the grain (both of barley of the 1964 harvest and malt) were treated with excess of cold acetone (distilled over concentrated sulphuric acid and potassium permanganate to oxidize any organic impurities) for 15 minutes at -15°C shaking every five minutes. The acetone was then removed by vacuum filtration at 5°C and thereafter the grain was exposed at room temperature for half an hour to drive off remaining traces of the acetone. The treated grain was then extracted, as usual, along with a similar weight of grain not treated with acetone and the Hb-ase activities of both the extracts were determined in the usual manner. The results (Table vii) showed that there was no particular advantage in treating the grain with acetone.

In view of the results obtained in the foregoing preliminary experiments, the final procedure adopted for the extraction of proteolytic enzymes from the malt was as follows. One hundred g. of the malt were finely ground by hand in a coffee mill and extracted with $2\frac{1}{2}$ - 3 volumes of 0.15 M sodium chloride solution containing 0.5% (w/v) L - cysteine - hydrochloride (neutralized to pH 7.0 with sodium hydroxide) for two hours in a mechanical shaker at 5°C. The slurry was strained through two layers of cheese cloth and the extract centrifuged at 19000 x g for 20 minutes. The supernatant fraction was dialysed for 16 hours in Visking dialysis tubing (32/32") against running cold water at 5°C to remove the salt and the low-molecular weight compounds. The dialysed extract was recentrifuged to remove materials which had sedimented during dialysis. The clear supernatant fraction (ca. 60% of the original extract volume) was lyophilized and the lyophilized preparation stored at -5°C.

The preparation was relatively stable and no appreciable loss in enzyme activity occurred over an extended period (ca. 6 months) of storage. This preparation was subsequently used for the fractionation of Hb-ase (see enzyme assay) by exclusion chromatography on Sephadex G-75 and ion-exchange chromatography on DEAE - cellulose.

(b) Protein Estimation.

Protein estimations were made by the biuret method as described by Layne (1957). To 1 ml. aliquots of protein solution were added 4 ml. of the biuret reagent and the solutions allowed to stand for 30 minutes at room temperature (18 - 20°C). The intensity of the color developed was read at 550 mμ against a control prepared by mixing 4 ml. of the biuret reagent and 1 ml. of water. The concentration of protein in the sample was determined by reference to a standard curve prepared with crystalline bovine albumin. All protein determinations were made in duplicate.

(c) Enzyme Assays.

The malt extract, prepared as above, hydrolysed Hb, BAPA, BAEE and BAA (see Reagents on p. 33). In addition it hydrolysed another substrate, an azo-glutenin, a wheat protein prepared according to a method described by Goad (1966) and kindly supplied by Mr. J. Bartlett of this Department.

The enzyme (s) which catalyse the hydrolysis of Hb, BAPA, BAEE, BAA and azo-glutenin have been referred to, in the text, as Hb-ase, BAPA-ase, BAEE-ase, BAA-ase and azo-glutenin-ase respectively.

(i) Hb-ase.

Hb-ase was assayed by a modification of the original method of Anson (1938) using commercial bovine haemoglobin as a substrate.

Haemoglobin Solution.

8 Because of the insolubility of commercial bovine haemoglobin, as found in these studies, in buffers of acidic pH, the haemoglobin substrate could not be prepared as described by Davis and Smith (1961) for the assay of proteolytic enzymes. Haemoglobin substrate was, therefore, prepared as follows: bovine commercial haemoglobin was suspended overnight in excess of 0.05M acetate buffer, pH 3.8, (the optimum pH of Hb-ase under the experimental conditions). The suspension was centrifuged to remove

the insoluble protein and an aliquot of the supernatant fraction dried at 100°C for one hour and its dry matter content determined. The rest of the haemoglobin solution was then suitably diluted with the same buffer to give a final substrate concentration of 1.5% (w/v). Merthiolate (Eli Lily & Company Ltd.), a preservative, was added at the rate of one mg. per 50 ml. of the solution and the substrate stored at 4°C. Storage at 4°C for up to three months had no deleterious effects on the substrate and caused no appreciable increase in the control.

Procedure.

Haemoglobin substrate (4 ml.) and the enzyme solution (1 ml.) were both equilibrated for 5 minutes in a constant temperature water bath at 40°C, the assay temperature. At zero time, the enzyme was added and gently mixed with the substrate. After appropriate times, the enzyme action was stopped by adding 5 ml. of 0.6M trichloroacetic acid (TCA). After thorough mixing, the mixture was held in the constant temperature water bath for 30 minutes to ensure complete coagulation of the undigested protein, and then filtered through Whatman No. 1 filter paper to obtain a clear filtrate. The extinction of the TCA - filtrate was measured at 280 mμ (Davis and Smith, 1961) against water. The reading of the control was substracted from that of the assay sample. The control was prepared with each assay in the following manner: enzyme solution was added directly to 5 ml. of the TCA and precipitated. Four ml. of the haemoglobin substrate solution were then mixed in and the mixture held at 40°C for 30 minutes after which it was filtered through Whatman No. 1 filter paper. Until otherwise stated, Hb-ase activity is expressed as change in extinction (Δ E) at 280 mµ per hour per mg. protein. Where column effluents were used in the assay, Hb-ase activity is expressed as change in extinction at 280 mµ per ml. per hour. A linear relationship was obtained when enzyme concentration was plotted against extinction (see Fig. 8).

When the effect of various inhibitors on Hb-ase activity was studied each inhibitor was dissolved in 0.05 M sodium acetate buffer, pH 3.8, the optimum pH of Hb-ase. All inhibitor solutions, therefore, had the same pH. The enzyme solution was prepared in such a way so as to contain in one ml. the appropriate concentrations of the inhibitor. The inhibitor solution was added directly to the enzyme solution which was then held at room temperature (18 - 20°C) for ten minutes before equilibrating in the water bath for the assay. For the control the inhibitor solution was replaced with water.

(ii) BAPA - ase.

BAPA - ase was determined by the original procedure of Erlanger et al. (1961) modified by Enari et al. (1963) for use at pH 8.6. 43.5 mg. DL-BAPA were dissolved in 1 ml. of the solvent dimethylsulphoxide and the solution made up to 100 ml. with 0.05M tris-(hydroxymethyl)-aminomethane - HCl (Tris-HCl) buffer of pH 8.6 to give a substrate concentration of 1×10^{-3} M. The substrate stock solution was stored in the incubator at 25°C. On prolonged storage, however, hydrolysis of the substrate occurred with a resultant increase in the blank. The substrate was, therefore, not stored more than a fortnight.

Procedure.

The reaction mixture consisted of 2.5 ml. of the substrate solution and 1 ml. of the enzyme solution, both equilibrated at 30°C for 5 minutes before the enzyme was added and mixed with the substrate. A suitable control was also set up with 2.5 ml. of the substrate and 1 ml. of water. After appropriate times, enzyme action was stopped by the addition of 1 ml. of 30% (v/v) acetic acid. The quantity of p-nitroaniline released (see Fig. 2) was estimated spectrophotometrically at 410 mμ. BAPA-ase activity is expressed as change in extinction (ΔE) at 410 mμ per hour per mg. protein or as change in extinction at 410 mμ per hour per ml. when BAPA-ase was assayed in the column effluents.

When the effect of various inhibitors on BAPA-ase was studied each inhibitor was dissolved in 0.05M Tris -HCl buffer, pH 8.6, the optimum pH of barley and malt BAPA-ase (Enari et al., 1963). As in the case of Hb-ase, the inhibitor solution was mixed with the enzyme solution which was then kept at room temperature for ten minutes before equilibrating in the water bath at 30°C for the assay. For the control the inhibitor solution was replaced with water.

(iii) BAEE -ase.

BAEE -ase was assayed by a modification of the spectrophotometric method of Schwert and Takenaka (1955) as used by Burger (1966). One ml. of the enzyme solution was added to 3.0 ml. of the substrate, 3×10^{-4} M BAEE in 0.05M phosphate buffer, pH 7.0. The control contained 3.0 ml. of 3×10^{-4} M BAEE in the same buffer. The reaction temperature was 30°C. BAEE-ase activity is expressed as optical extinction at 253 mμ per hour per ml. of enzyme solution. The hydrolysis of BAEE by malt enzyme, under the experimental conditions described, was proportional to the enzyme concentration (TableXIX).

(iv) BAA-ase.

BAA-ase was assayed by measuring the ammonia liberated from the BAA. 3 ml. of the substrate, 3×10^{-4} M BAA in 0.05M phosphate buffer pH 7.0 (Burger, 1966), were incubated with 1 ml. of the enzyme solution for one hour. The assay mixture was then quantitatively transferred to a quickfit distillation assembly and 5 ml. of 40% sodium hydroxide were added. The ammonia liberated was distilled into 2% boric acid solution containing a few drops of methyl red and methylene blue indicator (A.O.A.C., 1960) and titrated with standard hydrochloric acid (0.01N). For a control, the enzyme solution was substituted with water. The control was not ususually large in spite of treatment of the substrate with 40% sodium hydroxide. BAA-ase activity (see Table (XXI) is expressed as µg of ammonia liberated per hour per ml. of the fraction.

(v) Azo-glutenin-ase.

Azo-glutenin-ase was assayed by using a slightly modified method of Goad (1966). The assay mixture consisted of 1% (w/v) azo-glutenin (1 ml.); 0.1 M citric acid-phosphate buffer pH, 5.0 (0.2 ml.) and enzyme solution (1 ml.). The assay temperature was 40°C; the enzyme action being terminated after one hour by the addition of 2 ml. of 10% trichloroacetic acid (TCA). After the addition of TCA, the assay mixture was centrifuged at 3,000 r.p.m. for 15 minutes to obtain a clear supernatant fraction which was quantitatively transferred to 2N sodium hydroxide (2 ml.). After thorough mixing, the extinction of the mixture was read at

460 mμ (the azo-peptides released by the enzymic action absorb strongly at this wavelength at an alkaline pH). The reading for the control was substracted from those of the assay samples. The control was prepared by adding the enzyme solution directly to TCA. The buffer and the substrate were then mixed in and the mixture centrifuged and the clear supernatant fraction transferred to sodium hydroxide. No non-enzymic hydrolysis of the substrate occurred when it was kept in the water bath at 40°C, the assay temperature, up to two hours. Azo-glutenin-ase activity is expressed as change in optical extinction at 460 mμ for 15 - 120 minutes per mg. protein. A linear plot up to an assay time of sixty minutes was obtained when extinction was plotted against assay time (Fig. 7 lower curve).

(d) <u>Fractionation of Hb-ase.</u>

(i) <u>Exclusion Chromatography on Sephadex G-75.</u>

Sephadex G-75 was suspended for three days in excess of 0.05M sodium acetate buffer, pH 5.8, the eluting buffer. Fine particles of the gel were removed by repeated decantation. The gel slurry was packed into a 40 x 3 cm. glass column firmly plugged at the base with glass wool. The column was equilibrated with the eluting buffer for several hours before use and 125 mg. of the freeze-dried preparation dissolved in 3-4 ml. of the eluting buffer applied at the top of the gel bed. Elution was started when all the sample had entered the gel bed, and 3 ml. fractions were collected at a flow rate of 12 - 14 ml. per hour. The protein concentration in each fraction was estimated by measuring the extinction at 280 mμ (Warburg and Christian, 1941) against the eluting buffer. Hb-ase was assayed as described above using 1 ml. aliquots of the fractions. Exclusion chromatography was performed at room temperature (18 - 20°C).

(ii) Ion-Exchange Chromatography on DEAE - Cellulose.

The adsorbent powder was washed for packing into the column as described by Peterson and Sober (1962). The washed adsorbent was suspended in the starting buffer, 0.1M sodium orthophosphate, pH 7.0, and packed into a 70 x 1.5 cm. glass column which was equilibrated with the same buffer for 48 hours before use. 75 mg. of the freeze-dried preparations were dissolved in 3 ml. of the starting buffer and applied to the top of the adsorbent in the column. Elution was accomplished, at room temperature (18 - 20°C), in a step-wise fashion: first 125 ml. (5 ml. fractions) were collected by eluting with 0.1M sodium orthophosphate buffer, pH 7.0 and the second 125 ml. also in 5 ml. fractions by eluting with 0.5M sodium orthophosphate buffer, pH 4.0 (Enari and Mikola 1961) at a flow rate of 20 ml. per hour. The protein concentrations in the individual fractions and the Hb-ase activity of selected fractions were determined in the usual manner.

(e) Fractionation of Hb-ase and BAPA-ase.

(i) Ammonium Sulphate Fractionation.

The malt extract, prepared as described previously on page 37 (prior to lyophilysation), was precipitated at 4°C with appropriate concentrations of ammonium sulphate to give 0-20, 20-40, 40-60, 60-80 and 80-100% salt saturation according to a nomogram given by Green and Hughes (1955). The salt was added slowly, with mechanical stirring, to avoid local denaturation of the enzyme proteins. After appropriate additions of the salt, the extract was kept at 4°C for 20 minutes with shaking every five minutes. The proteins precipitated were recovered by centrifugation at 10,000 x g for 10 minutes, dissolved in small quantities of distilled water and dialysed for 20 hours in Visking dialysis tubing (32/32") against running tap water at 4°C. The non-diffusible material was recentrifuged and the supernatant fractions, in each case, were made to volume (25 ml.) quantitatively with distilled water. Suitable aliquots (1 ml.) of each fraction were taken to determine Hb-ase and BAPA-ase activities and for the determination of protein by the biuret method in the usual manner.

Reference to Table XXI shows that no sharp separation of the enzymes occurred on fractionation with ammonium sulphate and that most of the enzyme proteins were precipitated between 20 - 80% of salt saturation. Subsequently, the proteins in the extract were precipitated between 20 - 80% of ammonium sulphate saturation in the following manner. The extract was brought to 20% saturation with ammonium sulphate; the precipitate formed was recovered as usual and rejected. The supernatant fraction was adjusted to 80% saturation with ammonium sulphate and the proteins precipitated collected by centrifugation, dissolved in a minimal amount of water and dialysed, as before, against running tap water at 4°C. The non-diffusible material was recentrifuged and the clear supernatant fraction lyophilysed. The lyophilysed preparation was stored at -5°C and subsequently used for the fractionation of Hb-ase and BAPA-ase by exclusion chromatography.

(ii) Exclusion Chromatography on Sephadex G-100.

The dry Sephadex powder was suspended in excess of 1% (w/v) aqueous sodium chloride for seven days and fine particles of the gel removed by repeated decantations. The gel slurry, diluted with additional 1% sodium chloride, was poured into a 47 x 2 cm. glass column and the column equilibrated for 24 hours with the eluting buffer, 0.05M phosphate, pH 5.8, containing 2.5% (w/v) sodium chloride (Enari et al., 1963). 65 mg. of the freeze-dried preparation, dissolved in 3 ml. of buffer, were eluted and 5 ml. fractions collected as usual. The protein concentrations and Hb-ase and BAPA-ase activities were determined in the fractions using procedures described previously.

(c) SECTION 3: DEVELOPMENT OF PROTEASES IN GERMINATING BARLEY.

1. Materials.

Proctor barley, of the 1965 harvest, kindly supplied by Mr. T. Fergusson of Archibald Campbell, Hope and King Limited, Edinburgh, was used to study the development of proteases in the germinating barley grain.

2. Methods.

10 g. samples of barley (protein content 1.62%; Institute of Brewing, 1961) were steeped, at the germination temperature, in tap water for 24 hours with a change of steep after 12 hours. The grain was then air-rested for 12 hours and re-steeped in water for an additional 12 hours. At the end of steep, the moisture content of the grain, as determined by surface

drying of the whole grain at 100°C for 2 hours, was 43% . The steeped grain was grown at 13 - 15°C (relative humidity 85 - 90%) in a growth cabinet (G. Webster and Company, Glasgow) in 6 x $3\frac{1}{2}$" glass bottles with a screw-on lid into which a $\frac{1}{4}$" hole was drilled. This method of germination prevented excessive drying of the grain during the germination period. The bottles were shaken daily to prevent matting of the rootlets. At appropriate times, the bottles were removed and their contents homogenised at top speed with 3 volumes of cold 0.15 M sodium chloride solution (Morton, 1955) containing 0.5% (w/v) L-cysteine-hydrochloride neutralized to pH 7.0 with sodium hydroxide for 3 - 4 minutes in an MSE homogeniser and the homogenate extracted for two hours by shaking in a mechanical shaker at 4°C. When ungerminated barley was used, the grain was ground by hand first in a coffee mill and then extracted with 3 volumes of 0.15 M sodium chloride for two hours at 4°C. The homogenate, after extraction, was strained through two layers of cheese cloth to remove the debris and the extract centrifuged at 19,000 x g for 20 minutes. The supernatant fraction was made to volume (50 ml.) with water and 1 ml. aliquots taken for the determination of protein by the biuret method and for the assay of Hb-ase and BAPA-ase according to the procedures described earlier. Hb-ase and BAPA-ase activities are expressed as extinction units at 280 mμ and 410 mμ respectively per hour per g. barley.

RESULTS.

SECTION 1: AUTOLYSIS OF BARLEY AND MALT.

Comparison of Barley Varieties : Total Nitrogen and the Release of the Water-Soluble, Formol and Non-Protein Nitrogen.

1. Total Nitrogen Content of the Grain.

The total nitrogen content of the six barley varieties varied from a lowest of 1.29% for the Ymer barley to a highest of 1.65% for the Swallow barley (see Table VIII, Column 3, where the nitrogen content of the varieties is reported as mg. nitrogen per hundred g. dry barley). On the basis of the nitrogen content of the grain, the varieties may be divided into those having low nitrogen content viz. Proctor and Ymer and those having a high nitrogen content viz. Hunter, Maythorpe, Mentor and Swallow.

2. Water-Soluble Nitrogen.

The release of Water-Soluble nitrogen in all the six barley varieties autolysed at 40°C for times ranging from 15 minutes to 4 Hours is given in Table VIII and Fig. 3. These results show that the release of Water-Soluble nitrogen was higher for the varieties Mentor and Swallow, which had a high nitrogen content and lower for the varieties Proctor and Ymer which had a low nitrogen content. Although the varieties Hunter and Maythorpe had the same nitrogen content (1.29% each) slightly more nitrogen was solubilised when the variety Hunter was uatolysed for 15, 30, 60, 120 and 240 minutes than when the variety Maythorpe was autolysed for the same periods of time. The Water-Soluble nitrogen formed between 8-20%

of the total grain nitrogen (Table VIII, Column 7 and Fig. 4) and showed little variation amongst the varieties regardless of the original nitrogen content of the grain.

3. Formol Nitrogen.

Table VIII, Column 5 and Fig. 3 show an estimate of the Formol nitrogen released from the varieties autolysed for 15, 30, 60, 120 and 240 minutes. The release of Formol nitrogen, in general, followed the same pattern as the release of the Water-Soluble nitrogen i.e. it was higher for the varieties Swallow, Mentor and Hunter, which had a high nitrogen content and lower for the variety Proctor, which had a low nitrogen content. The variety Ymer, however, inspite of a low nitrogen content, gave a higher estimate of the Formol nitrogen as compared to the variety Proctor, although these two varieties had the same nitrogen content. As percent of the Water-Soluble nitrogen, the Formol nitrogen constituted between 10 - 18% (Table VIII, Column 8 and Fig. 4) and varied little between the varieties apart from a small rise, particularly at the 4 hours autolysis time, in the case of the variety Ymer.

4. Non-Protein Nitrogen.

Table VIII, Column 6 and Fig. 3 show the release of the Non-Protein nitrogen (or Lundin's fraction C) in the six barley varieties autolysed for various periods of time. A comparison of the release of the Formol and the Non-Protein nitrogen shows that the Non-Protein nitrogen, in most cases, was twice the Formol nitrogen. This was so because the Formol nitrogen estimated the amino nitrogen contributed by the amino acids as well as by the low-molecular weight peptides, while Non-Protein nitrogen probably contained, in addition to amino acids and low-molecular weight peptides, polypeptides and other nitrogenous compounds present in the autolysate which were not precipitated by the phosphomolybdic acid, the protein precipitant employed. As percent of the Water-Soluble nitrogen, the Non-Protein nitrogen constituted between 22-35% (Table VIII, Column 9). Again, no varietal differences were discernible and the release of the Non-Protein nitrogen, in general, like Formol nitrogen showed the same trends as the release of the Water-Soluble nitrogen.

In Table IX are given the Water-Soluble, Formol, Non-Protein nitrogen and the related data for the four hours autolysis time of the varieties which are tabulated in order of increasing nitrogen content of the grain. These data have been extracted from Table VIII and are set out separately to show the maximum proteolysis which occurred under the experimental conditions described earlier. These data show that inspite of a significant variation in the nitrogen content of the grain, the varieties could not be distinguished on the basis of proteolytic activity as measured by the release of the Water-Soluble, Formol and Non-Protein nitrogen. The Water-Soluble nitrogen as percent of the Total nitrogen was substantially the same (circa 20%) in each case except in the variety Maythorpe for which the corresponding figure was 18.6%. Again a close similarity of the varieties was apparent when the Formol and Non-Protein nitrogen were calculated as percent of the Water-Soluble Nitrogen. (Fig.4).

Effect of Inhibitors and a Activator on Proteolysis in Barley and Malt.

Inhibitors.

1 Potassium Bromate.

The effect of 125, 500 and 2,000 mg/L concentrations of potassium bromate on the release of the Water-Soluble, Formol and Non-Protein nitrogen, both in barley and malt autolysed for 15, 30, 60, 120 and 240 minutes is shown in Table X. . Except for a noticeable inhibitory effect, which increased with increase in the concentration of the inhibitor, on the Water-Soluble and Non-Protein nitrogen estimated from the 240 minutes autolysis of malt, potassium bromate did not show any consistent inhibitory effect on the nitrogen fractions estimated from the other autolysis times. On the other hand, in most cases, the release of the Water-Soluble, Formol and Non-Protein nitrogen was slightly enhanced, particularly when the inhibitor was present at the highest concentration, when the grain was autolysed with water containing potassium bromate than when the grain was autolysed with water alone (control).

2. Phenylmercury acetate.

Phenylmercury acetate, a specific SH enzyme inhibitor, when applied at the three concentrations of 125, 500, and 2,000 mg/L inhibited the production of the Water-Soluble, Formol and Non-Protein nitrogen both in barley and malt autolysed for 15, 30, 60, 120 and 240 minutes (Table XI). The inhibition of the nitrogen fractions by phenylmercury acetate was, in most cases, consistent and increased with increase in the concentration of the inhibitor.

3. Iodoacetic Acid.

The effect of iodoacetic acid, an alkylating agent which inhibits the SH enzymes irreversibly, on the production of the Water-Soluble, Formol and Non-Protein nitrogen was studied only at one concentration i.e. 500 mg/L. The results are given in Table

XII . Both in barley and malt, autolysed for 15, 30, 60, 120 and 240 minutes, iodoacetic acid inhibited the production of all three nitrogen fractions estimated.

Activator.

1. Thioglycollic Acid.

Table XIII shows the results of the effect of the three concentrations 125, 500 and 2,000 mg/L of the reducing agent, thioglycollic acid, on proteolysis in barley and malt. These data indicate that thioglycollic acid activated the production of the Water-Soluble, Formol and Non-Protein nitrogen both in barley and malt autolysed for 15, 30, 60, 120 and 240 minutes. Although the activation of the nitrogen fractions was, in most cases, small, nevertheless it was consistent and, in general, increased with increase in the concentration of thioglycollic acid.

SECTION 2: FRACTIONATION OF PROTEOLYTIC ENZYMES OF MALT.

Although the malt extract, prepared as described earlier, hydrolysed a number of substrates (see Reagents on p. 33 under EXPERIMENTAL Section), for the fractionation of malt proteolytic enzymes only two substrates were used. These were haemoglobin (Hb), which, being a non-specific substrate, assayed the total proteolytic activity of the malt, and a synthetic substrate, N- α - benzoyl - L - arginine - p - nitroanilide (BAPA), specific for a "trypsin-like" enzyme reported by Enari et al. (1963) to be present in water extracts of barley and malt. The results, reported in this section are, therefore, mainly those of studies conducted on Hb-ase and BAPA-ase, the enzymes which catalyse the hydrolysis of Hb and BAPA.

1. Studies on Hb-ase.

Experiments were first conducted to establish optimum conditions for the assay of Hb-ase. The optimum conditions investigated were, the pH, temperature, time of assay, substrate concentration and the effect of enzyme concentration on the rate of hydrolysis of the haemoglobin substrate. The final procedure adopted for the assay of Hb-ase described earlier (see EXPERIMENTAL Section) was largely based on the results obtained from these experiments.

(a) Effect of pH on the Hb-ase Activity of a Freeze-dried Extract of Malt.

The haemoglobin substrate was prepared as described earlier in three different buffers to cover a pH range of 3 to 9. The buffers used were:

pH	3.0	— 6.0	0.05M	citrate
pH	7.0	— 8.0	0.05M	phosphate
pH	8.0	— 9.0	0.05M	borate

As can be seen in Table XIV and Fig. 5 a single peak of enzyme activity was obtained with pH optimum close to 3.8. A sharp decrease (ca. 23%) in the Hb-ase activity occurred with a relatively small rise in the substrate pH from 3.8 to 4.0. Thereafter the Hb-ase activity steadily decreased as the pH of the substrate was increased. At pH 6.0, the enzyme had lost over ca. 85% of its activity at pH 3.8.

At pH 7.0, the enzyme activity increased inspite of the fact that a slight precipitation of the substrate occurred, the isoelectric point of haemoglobin being at pH 6.8 (Dawson et al., 1959). Although the evidence is not conclusive, it is possible that another enzyme with a pH optimum at neutrality was involved at this pH.

It is not known whether the buffer ions affected the Hb-ase activity as this effect was not investigated.

(b) Effect of Temperature on the Rate of Deactivation of Hb-ase.

The enzyme solution was held for five minutes in a constant temperature water bath adjusted to various temperatures. Thereafter the enzyme solution was cooled to room temperature and then used for the assay. Table XV and fig. 6 show the effect of temperature on the rate of deactivation of Hb-ase under the experimental conditions described. Although the enzyme (s) appeared to be deactivated at 30°C, this is probably an experimental error. No deleterious effects on enzyme activity were noticeable at 35 to 40°C. The deactivation of Hb-ase started at 45°C and at 60°C 78% of the enzyme activity was lost.

In all subsequent assays, the substrate and the enzyme solution were held at 40°C for five minutes before the enzyme was added to the substrate. Since the assay time was one hour, it is possible that some denaturation of the enzyme occurred during the assay.

(c) Effect of Time of Assay on the Rate of Hydrolysis of Hb.

Table XVI and fig. 7 (upper curve) show the hydrolysis of 1.5% haemoglobin at pH 3.8 and 40°C by malt enzyme (s) for times ranging from 10 minutes to 3 hours. The rate of hydrolysis of haemoglobin was substantially linear up to sixty minutes, after which it gradually fell off. The graph of time of assay vs the rate of hydrolysis of the substrate, obtained under the experimental conditions described, was typical of the progress curve of an enzyme reaction. To ensure that maximal activity was measured a one hour assay period was selected for subsequent assays.

(d) Effect of Enzyme Concentration on the Rate of Hydrolysis of Haemoglobin.

The graph of the rate of hydrolysis of 1.5% haemoglobin solution, pH 3.8, against enzyme concentration (samples of freeze-dried malt extract containing 1 to 5 mg. protein) was linear (Table XVII and fig. 8). The linear plot obtained suggested that the substrate concentration employed (1.5%) was sufficient to sustain under the experimental conditions maximal enzyme activity.

(e) Effect of Substrate Concentration on the Hb-ase Activity.

Table XVIII and fig. 9 show the effect of the haemoglobin concentration on its rate of hydrolysis by malt enzyme (s) at pH 3.8 and 40°C. The substrate concentrations studied were 0.5, 1.0, 1.5, 2.0 and 2.5%. The maximum Hb-ase activity was obtained at a substrate concentration of 1.0 to 1.5%. At higher substrate concentrations inhibition of the Hb-ase occurred, the inhibition being ca. 18% at a substrate concentration of 2.5%. For subsequent enzyme assays, the substrate concentration employed was 1.5%. This concentration was selected to avoid any limiting effect of substrate concentration on the Hb-ase activity; the slight inhibition (1.5%) which occurred at this concentration being considered within the range of experimental error of the assay.

(2) Studies on BAPA-ase.

The assay conditions (pH, temperature, and substrate concentration, etc.) employed for the determination of BAPA-ase were those described by Enari et al. (1963) and subsequently used by Suolinna et al. (1965) and Burger (1966). Under these experimental conditions, the hydrolysis of 2.5 ml. of 1×10^{-3} M α - benzoyl - L - arginine - p - nitroanilide (BAPA) by malt enzyme was linear with the assay time (Table XX and Fig. 10 lower curve) up to 90 minutes. A linear plot was also obtained up to an assay time of 60 minutes (Table XX and Fig. 10 upper curve) when the same concentration of BAPA substrate was hydrolysed, under identical assay conditions, with one ml. of water containing 100 μg. of commercial trypsin (Koch-Light Laboratories Ltd.). The plot of the rate of hydrolysis of BAPA vs enzyme concentration (1 to 5 mg. protein of the freeze-dried extract of malt) did not, however, approach linearity (Table XIX), a finding similar to that reported by Suolinna et al. (1965).

(3) Fractionation of Hb-ase.

(a) Exclusion Chromatography on Sephadex G-75.

Fig. 11 shows the elution diagram of 125 mg. of the freeze-dried preparation obtained from the malt on Sephadex G-75 with 0.05M sodium acetate buffer, pH 5.0. As can bee seen, while the substances absorbing at 280 mμ resolved into four easily distinguishable peaks, all the Hb-ase activity appeared in a single peak which coincided with one of the two main 280 mμ absorption peaks between the elution volumes of 100 and 175 ml. When the exclusion chromatography of the freeze-dried malt extract was repeated, under identical conditions of elution, using a longer column (70 cm. long x 1.5 cm. diameter as compared with a column 40 cm long x 3 cm. diameter previously used) an essentially similar pattern of results was obtained to that shown in fig.11

The main 280 mμ peak obtained between elution volumes of 210 to 270 ml. apparently contained materials with strong absorption at 280 mμ. These fractions contained no Hb-ase activity whatsoever, and did not give, in most cases, a positive biuret reaction suggesting that no detectable quantities (by the biuret method) of peptide materials were present. In a subsequent elution, the appropriate fractions (elution volumes 100 to 175 ml.) containing all the Hb-ase activity were pooled, dialysed against distilled water at 5°C, for 16 hours in Visking dialysis tubing (32/32"), the non-diffusible material centrifuged and the clear supernatant fraction freeze-dried. The yield of the freeze-dried preparation was 102 mg. (ca. 82% of the original 125 mg. eluted).

This preparation was redissolved in 3 - 4 ml. of the eluting buffer 0.05M sodium acetate, pH 5.0 and rechromatographed on the same column (40 cm. long x 3 cm diameter) as described earlier. Again all the Hb-ase activity was eluted in a single peak coinciding with one of the two 280 mμ peak. On rechromatography, the 210 to 270 ml. absorption peak of fig.11 was greatly reduced.

(b) Ion-Exchange Chromatography on DEAE-Cellulose.

Fig12 shows the step-wise elution on diethylaminoethyl (DEAE) - cellulose of 75 mg. of the freeze-dried extract of malt containing a known quantity of Hb-ase activity. The first 125 ml., in 5 ml. fractions, were eluted with 0.1M orthophosphate buffer, pH 7.0 and the second 125 ml., also in 5 ml. fractions, with 0.5M orthophosphate buffer, pH 4.0. While the proteins were eluted by both the buffers and appeared in two peaks, all the Hb-ase activity (% recovery ca. 94) appeared in the peak eluted with 0.1M orthophosphate buffer, pH 7.0. Since most of the Hb-ase activity was already accounted for and the proteins eluted with the second buffer 0.5M orthophosphate, pH 4.0 contained no Hb-ase activity at all, no further elutions were carried out.

Latterly, an attempt was made to employ gradient elution with sodium acetate buffer, pH 5.0 with a buffer molarity of 0.01 M to 0.05M. The Hb-ase activity again appeared in a single peak, though in this case the concentration gradient was not completely satisfactory.

(4) **Fractionation of Hb-ase and BAPA-ase.**

(a) **Ammonium Sulphate Fractionation.**

Because of the lack of separation of malt enzymes on Sephadex G-75 and DEAE-cellulose, fractionation of these enzymes was attempted by ammonium sulphate precipitation and exclusion chromatography on Sephadex G-100 using, in addition to haemoglobin as substrate, α - benzoyl - L - arginine - p - nitroanilide (BAPA) for the enzyme assays. During the fractionation with ammonium sulphate, another synthetic substrate, benzoylarginamide (BAA) (fig.[2]) specific for trypsin was employed.

Results of fractionation of the malt extract, prepared as described earlier, with increasing concentrations of ammonium sulphate are presented in Table XXI where the enzyme activities (of Hb-ase, BAPA-ase and BAA-ase) and the concentration of protein are given as percentages of the total activity of these enzymes and of total protein in the unfractionated extract. As can be seen in the Table, maximum activities of the Hb-ase, BAPA-ase and BAA-ase were concentrated in the fraction precipitated between 40 to 60% ammonium sulphate saturation. As this salt concentration a maximum precipitation of the protein, as measured by the biuret method of protein estimation, also occurred. The percent total recoveries of the protein, Hb-ase and BAPA-ase were 74.0, 73.0 and 73.5% respectively and were remarkably similar, the percent total recovery of BAA-ase being only 52.3. The low recovery of BAA-ase may be attributed to denaturation of this enzyme (BAA-ase and BAPA-ase may be two separate enzymes but having the same substrate specificity) in the salting out procedure. Alternatively, it may also be due to low sensitivity of the method employed for the estimation of ammonia released by enzymic action.

Table XXI also shows that most of the Hb-ase activity was precipitated, under the experimental conditions described, between 40 to 80% ammonium sulphate saturation and that the BAPA-ase precipitated between 20 to 80% ammonium sulphate saturation. This concentration of ammonium sulphate also precipitated most of the non-enzymic proteins of the malt extract. Since there was no sharp separation of the different enzymes in the various fractions, in subsequent experiments the 20 to 80% ammonium sulphate saturation fraction was used for chromatography.

(b) Exclusion Chromatography on Sephadex G-100.

Fig. 13 shows the elution diagram of 65 mg. of the freeze-dried extract, precipitated between 20 to 80% ammonium sulphate saturation, on Sephadex G-100 eluted with 0.05M phosphate buffer, pH 5.8, containing 2.5% (w/v) sodium chloride. The salt was added to the eluant to minimise adsorption, if any, of the proteins to the Sephadex (Sober et al., 1965). The elution pattern obtained revealed two protein regions: the protein peak which coincided with most of the Hb-ase and BAPA-ase activities, and a large protein peak which contained a little of the Hb-ase activity but none of the BAPA-ase activity. Again because of the poor resolution it was not possible to separate the enzymes, nor the enzymes from the cotaminating proteins.

Enzyme Inhibitor Studies.

Experiments were conducted to determine the extent of inhibition of the Hb-ase and BAPA-ase activities of malt extract precipitated between 20 to 80% ammonium sulphate saturation by certain specific and non-specific inhibitors. All the inhibitors were tested at the optimum pH of each enzyme (3.8 for the Hb-ase; 8.6 for the BAPA-ase) and the procedures for employing inhibitors have been described earlier under the respective enzyme assays. The specific inhibitors used were the alkylating agents, iodoacetamide and N-ethylmaleimide, the mercury derivatives, phenylmercury acetate, p -chloromercuriphenylsulphonic acid (only tested on the Hb-ase as it was not soluble at alkaline pH's), p - hydroxymercuribenzoate (only tested on the BAPA-ase as it was not soluble at acidic pH's) and phenylmethylsulphonylfluoride which like the organophosphorus compound diisopropylphosphofluoridate inhibits those enzymes in which serine is involved in the active centre (for example, trypsin and chymotrypsin). In addition to these, potassium bromate was used. This reagent has been employed by Macey and Stowell (1957) to restrict the proteolytic activity of barley.

Effect of Various Inhibitors on the Hb-ase and BAPA-ase Activities of Freeze-dried Extract of Malt.

(i) Potassium Bromate.

Potassium bromate when used at the concentration of 1×10^{-4} or 1×10^{-3} M had no inhibitory effect on the Hb-ase activity of malt (Table XXII). At a bromate concentration of 1×10^{-2} M, however, the Hb-ase activity was inhibited by about one-third. With a further tenfold increase in bromate concentration, the inhibition of the Hb-ase reached 59%.

BAPA-ase was slightly inhibited (7.7%) only by the lower concentration (1×10^{-3} M) of potassium bromate. At the higher concentration of potassium bromate (1×10^{-2} M), however, the BAPA-ase activity was the same as that with the control (Table XXII).

(ii) Iodoacetamide.

None of the iodoacetamide concentrations employed showed any inhibitory effect on the Hb-ase activity (Table XXIII). On the other hand, the enzyme activity was slightly increased on the addition of various concentrations of the alkylating agent. The BAPA-ase activity was also increased by two concentrations (1×10^{-3} and 1×10^{-2} M) of iodoacetamide (Table XXIII). At a concentration of 1×10^{-1} M, however, iodoacetamide inhibited the BAPA-ase activity of malt by 7%.

(iii) N-ethylmaleimide.

Only at the highest concentration employed (1×10^{-2} M) did N-ethylmaleimide cause an inhibition (18%) of the Hb-ase (Table XXIV). At the other three concentrations, the inhibition of the Hb-ase was negligible or possibly even negative.

BAPA-ase was not inhibited by this reagent when used at a concentration of 1×10^{-4} M. Inhibition of the BAPA-ase, however, occurred at higher concentrations, being 13.4% at a concentration of 1×10^{-3} M and 53.4% at a N-ethylmaleimide concentration of 1×10^{-2} M (Table XXIV).

(iv) Phenylmercuryacetate.

This inhibitor was tested only on the Hb-ase as it was not soluble in buffers of alkaline pH's. Phenylmercuryacetate inhibited the Hb-ase activity at all the concentrations employed (Table XXV), the inhibition increasing with increase in the concentration of phenylmercuryacetate. At a phenylmercuryacetate concentration of 1×10^{-6} M, the inhibition of the Hb-ase was small (2.8%). At a concentration of 1×10^{-2} M, over 75% of the Hb-ase activity was inhibited. At comparable concentrations, under the experimental conditions described, phenylmercuryacetate proved to be the most effective inhibitor of the Hb-ase.

(v) p-Chloromercuriphenylsulphonic Acid.

At a relatively low concentration, e.g. 1×10^{-6} or 1×10^{-5} M, this reagent showed no inhibitory effect on the Hb-ase, the effect being rather the opposite particularly at the latter concentration. The inhibition of the Hb-ase occurred at higher concentrations, being 3% at a concentration of 1×10^{-4} M, 17% at a concentration of 1×10^{-3} M and 24.6% at a concentration of 1×10^{-2} M (Table XXVI).

(vi) p-Hydroxychloromercuribenzoate.

This inhibitor being soluble only at an alkaline pH, was tested for its effect on the BAPA-ase activity only. All the three concentrations employed inhibited BAPA-ase acitivity, the inhibition being 14.3% at a concentration of 1×10^{-5} M, 50% at a concentration of 1×10^{-4} M and 64.3% at a concentration of 1×10^{-3} M (Table XXVII).

(vii) Ethylenediaminetetraacetic Acid (EDTA).

Results of the effect of three concentrations of EDTA on the Hb-ase and BAPA activities were, in general, similar. All three concentrations of the chelating agent activated the Hb-ase and BAPA-ase, the activation of Hb-ase at a concentration of 1×10^{-1} M being slightly smaller than the activation obtained at a concentration of 1×10^{-2} M. In the case of BAPA-ase the activation with 1×10^{-1} or 1×10^{-2} M concentrations of EDTA was exactly the same (Table XXVIII).

(viii) Phenylmethylsulphonylfluoride.

This reagent, being a specific inhibitor of trypsin, was employed to study its effect on the BAPA-ase activity of malt and commercial trypsin (concentration 25 μg/ml) used in the hydrolysis of BAPA. The results are presented in Table XXIX. Phenylmethylsulphonylfluoride showed no inhibitory effect whatsoever on the BAPA-ase activity of malt when used at three concentrations of 1×10^{-5}, 1×10^{-4} and 1×10^{-3} M. When used at the same concentrations phenylmethylsulphonylfluoride, however, considerably inhibited the activity of commercial trypsin, the inhibition being ca. 28% at a concentration of 1×10^{-3} M. The inhibition with the other two concentrations was small but increased with increase in the concentration of the inhibitor.

SECTION 3: DEVELOPMENT OF PROTEASES IN GERMINATING BARLEY.

Table XXX and fig. 14 show the development of the Hb-ase and BAPA-ase activities of germinating barley in conjunction with the protein estimated by the biuret method. It can be seen that both the Hb-ase and BAPA-ase activities slightly increased on steeping, after which they were constant till the second day of germination. Thereafter the Hb-ase and BAPA-ase activities steadily increased, reaching a maximum, in each case, on the fifth day of germination, after which little change in the enzyme activities occurred till the ninth day of germination. The increase in the activities of Hb-ase and BAPA-ase on germination was therefore, under the experimental conditions described, threefold, and substantially the same in each enzyme. Although both the enzymes reached maximum activites on the fifth day of germination, the rate of increase of the Hb-ase was highest on the fourth day of germination and that of the BAPA-ase was highest on the fifth day of germination.

The protein content of the ungerminated and steeped barley was the same but decreased afterwards till the second day of germination. Thereafter the protein content steadily increased as the germination proceded and reached a maximum, like the Hb-ase and BAPA-ase activities, on the fifth day of germination, the increase in the protein content on germination being one and a half fold. After the fifth day of germination, the protein content decreased till the seventh day after which it remained constant till the ninth day of germination.

Table II Titration of 15 minutes autolysate (50 ml.) obtained from Proctor barley (125 g) of the 1962 harvest with 0.05 N sodium hydroxide.

No. of Autolysate.	Estimated pH of Inflection.	Estimated titre*
1	8.30	4.9
2	8.45	5.2
3	8.60	5.2
4	8.50	5.2
5	8.60	5.1
6	8.60	4.9
Mean ± S.E. (M)	8.51 ± 0.05	5.1 ± 0.06

* mls. 0.05 N sodium hydroxide per 50 ml. of the autolysate.

Table III Titration of 15, 30, 60, 120 and 240 minutes autolysates (50 ml.) obtained from Ymer barley (125 g) of the 1962 harvest with 0.05 N sodium hydroxide.

Autolysis Time (min.)	Estimated pH of Inflection.	Estimated titre*
15	8.50	5.8
30	8.55	7.2
60	8.65	10.6
120	8.60	13.6
240	8.40	16.1
Mean ± S.E.(M)	8.54 ± 0.05	

* mls. 0.05 N sodium hydroxide per 50 ml. of the autolysate.

Table IV **Titration of 15, 30, 60, 120 and 240 minutes autolysates (50 ml.) of six barley varieties (125 g. each) of the 1963 harvest with 0.05 N sodium hydroxide.**

AUTOLYSIS TIME (MINUTES)

Variety	15		30		60		120		240	
	pH of infl.	Titre.	pH of Infl.	Titre	pH of Infl.	Titre	pH of Infl.	Titre	pH of Infl.	Titre
Hunter	8.35	4.8	8.60	8.0	8.65	8.2	8.50	13.0	8.35	16.0
Maythorpe	8.50	5.0	8.65	7.6	8.75	10.7	8.55	14.5	8.47	17.2
Mentor	8.60	6.8	8.70	8.2	8.60	9.0	8.55	15.3	8.40	16.1
Proctor	8.85	6.7	8.70	7.6	8.60	11.9	8.50	11.8	8.42	15.6
Swallow	8.70	6.1	8.50	10.0	8.70	12.3	8.65	17.5	8.40	18.1
Ymer	8.30	4.0	8.65	6.8	8.56	9.8	8.45	11.6	8.35	14.2

Mean = 8.55 ± 0.02

Titre: 0.05N NaoH/50 ml. of autolysate.

General mean of Tables II, III and IV = 8.53

Table V : <u>The Hb-ase activity of malt (10g.) extracted with distilled water, 0.15 M sodium chloride and 5% (w/v) potassium sulphate.</u>

Assay Time (min.)	Hb-ase activities*		
	Water	0.15M NaCl	5% K_2SO_4
10	None	0.06	0.07
20	0.05	0.14	0.12
30	0.07	0.16	0.19
40	0.10	0.22	0.24
50	0.13	0.25	-
60	0.14	0.31	0.34

* Activities are expressed per one ml. of the final extract volume (50 ml.)

Table VI: <u>Effect of 0.5% (w/v) L-Cysteine-hydrochloride acid on the Hb-ase activity of malt (10 g.) extracted with 0.15 M sodium chloride solution.</u>

Assay Time (min.)	Hb-ase activities*	
	No L-cysteine-HCl added	0.5% L-cysteine-HCl added
10	0.06	0.07
20	0.14	0.14
40	0.22	0.26
60	0.31	0.37

*Activities are expressed per one ml. of the final extract volume (50 ml.)

Table VII: Comparison of Hb-ase activity of the acetone treated (AT) and not treated (NT) barley of the 1964 harvest and malt (25 g. each).

Assay Time (min.)	Hb-ase activities*			
	Barley		Malt	
	AT	NT	AT	NT
5	None	None	0.02	0.04
10	0.01	0.01	0.12	0.18
20	0.02	0.03	0.28	0.30
40	0.04	0.05	0.54	0.57
60	0.11	0.09	0.83	0.80

* Activities are expressed per one ml. of the final extract volume (50 ml.)

Table VIII : Autolysis of barley: Release of the Water-Soluble, Formol and Non-Protein nitrogen.

Barley variety	Autolysis Time (min.)	mg. per 100 g. dry barley				% of TN	% of WSN	
		TN	WSN	FN	NPN	WSN	FN	NPN
Hunter		1500						
	15		128.6	13.7	29.0	8.6	10.7	225
	30		157.6	18.2	39.0	10.5	11.6	24.7
	60		205.1	29.7	50.2	13.7	14.5	24.5
	120		253.7	34.8	67.4	16.9	13.7	26.6
	240		306.3	54.3.	90.4	20.4	17.7	29.5
Maythorpe		1500						
	15		111.7	11.8	32.5	7.4	9.9	29.1
	30		139.7	20.4	42.5	9.3	14.7	30.4
	60		176.8	26.7	51.4	11.8	15.1	29.0
	120		236.6	33.2	65.0	15.8	14.0	27.4
	240		279.3.	46.9	96.3	18.6	16.8	34.5.

TN = Total nitrogen

WSN = Water-Soluble nitrogen

FN = Formol nitrogen

NPN = Non-Protein nitrogen

Table VIII (Contd.)

Mentor		1550						
	15		134.4	19.0	37.2	8.7	14.1	27.6
	30		170.3	22.7	47.9	11.0	13.3	28.1
	60		219.2	36.2	57.9	14.1	16.5	26.4
	120		262.8	39.4	79.8	17.0	15.0	20.4
	240		338.3	55.0	102.8	21.8	16.3	30.4
Proctor		1360						
	15		112.2	12.2	29.5	8.2	10.9	26.3
	30		150.1	20.0	39.6	11.1	13.2	26.2
	60		186.7	30.2	49.6	13.7	18.3	26.5
	120		233.8	35.4	59.7	17.2	15.1	25.5
	240		272.1	39.1	80.4	20.0	14.3	29.5

Table VIII (Contd.)

Swallow		1650						
	15		127.7	15.5	31.3	7.7	12.1	24.5
	30		170.1	23.2	45.5	10.3	13.6	26.7
	60		210.9	34.6	58.5	12.8	16.4	27.7
	120		297.9	39.4	81.0	18.0	39.9	27.5
	240		333.7	59.8	108.2	20.2	17.9	32.4
Ymer		1290						
	15		122.3	14.2	24.2	9.5	11.6	19.8
	30		139.0	19.4	36.6	10.8	14.0	26.3
	60		180.3	36.6	47.3	14.0	20.3	26.2
	120		227.7	42.1	68.6	17.3	16.3	30.1
	240		268.0	49.1	93.4	20.8	18.3	34.8

Table IX : The release of the Water-Soluble, Formol and Non-Protein nitrogen in six barley varieties autolysed for 4 hours (data extracted from Table VIII and tabulated in order of increasing nitrogen content of the varieties).

Barley variety	mg. per 100 g. dry barley				% of TN	% of WSN	
	TN	WSN	FN	NPN	WSN	FN	NPN
Ymer	1290	268.0	49.1	93.4	20.8	18.3	34.8
Proctor	1360	272.1	39.1	80.4	20.0	14.3	29.5
Hunter	1500	306.3	54.3	90.4	20.4	17.7	29.5
Maythorpe	1500	279.3	49.9	96.3	18.6	16.8	34.5
Mentor	1550	338.3	55.0	102.8	21.8	16.3	30.4
Swallow	1650	333.7	59.8	108.2	20.2	17.9	32.4

TN = Total nitrogen

WSN = Water-Soluble nitrogen

FN = Formol nitrogen

NPN = Non-Protein nitrogen

Table X : Effect of various concentrations of potassium bromate on the release of the Water,-Soluble, Formol and Non-Protein nitrogen in barley and malt.

Autolysis Time (min.)	Inhibitor conc. mg/L*	BARLEY mg. per 100 g. WSN	FN	NPN	MALT mg. per 100 g. WSN	FN	NPN
15	0	118.8	12.3	27.8	436.8	79.5	190.4
	125	121.0	12.0	31.8	450.8	80.6	199.5
	500	130.0	13.4	31.2	428.4	78.4	165.7
	2000	126.8	10.1	29.2	436.8	79.5	188.5
30	0	156.4	18.5	35.1	534.8	86.9	232.0
	125	158.7	14.6	37.1	534.8	90.7	227.5
	500	158.2	18.1	38.4	491.1	97.4	214.4
	2000	165.0	20.2	36.4	532.0	95.2	217.7
60	0	201.6	22.4	46.4	616.0	119.8	288.6
	125	207.6	23.5	51.7	588.0	106.4	267.1
	500	198.1	22.4	53.0	571.2	109.8	263.2
	2000	203.8	21.8	47.7	644.0	107.5	278.2

* The corresponding approximate mM concentrations of 125, 500 and 2,000 mg/L potassium bromate are 0.7, 3.0 and 12.0 respectively.

WSN = Water-Soluble nitrogen

FN = Formol nitrogen

NPN = Non-Protein nitrogen

Table X (Contd.)

120	0	261.5	30.2	67.0	700.0	146.7	351.6
	125	241.1	31.9	66.3	630.0	125.4	325.0
	500	238.7	32.5	64.3	599.2	131.0	282.7
	2000	262.7	31.6	68.3	660.8	140.	312.0
240	0	302.6	42.6	88.8	806.4	185.9	435.5
	125	304.9	46.7	88.2	753.2	166.9	406.2
	500	294	41.4	86.8	744.4	174.4	390.0
	2000	304.9	41.4	88.8	700.0	168.0	377.0

Table XI : **Effect of various concentrations of phenylmercury acetate on the release of the Water-Soluble, Formol and Non-Protein nitrogen in barley and malt.**

Autolysis Time (min.)	Inhibitor conc. mg/L*	BARLEY mg. per 100 g.			MALT mg. per 100 g.		
		WSN	FN	NPN	WSN	FN	NPN
15	0	118.8	12.3	27.8	436.8	79.5	190.4
	125	121.6	14.0	23.1	431.8	85.1	201.5
	500	117.6	12.9	23.8	375.2	76.2	175.5
	2000	95.3	12.5	22.5	367.9	73.9	185.2
30	0	156.4	18.5	35.1	534.8	86.9	232.0
	125	154.7	17.4	31.1	502.9	83.0	221.0
	500	131.6	19.0	30.1	478.8	80.5	214.5
	2000	121.0	16.9	28.4	431.2	75.1	211.9
60	0	201.6	22.4	46.4	616.0	119.8	288.6
	125	198.7	22.4	42.4	568.4	115.4	273.0
	500	156.4	21.8	39.7	561.7	98.6	251.5
	2000	147.6	21.3	35.7	540.4	93.0	243.7

* The corresponding approximate mM concentrations of 125, 500 and 2,000 mg/L phenylmercuryacetate are 0.4, 1.5, and 5.9 respectively.

WSN = Water-Soluble nitrogen

FN = Formol nitrogen

NPN = Non-Protein nitrogen

Table XI (Contd.)

120	0	261.5	30.2	67.0	700.0	146.7	351.6
	125	239.2	28.2	66.3	631.7	137.8	325.0
	500	214.7	26.9	67.6	594.7	119.8	320.4
	2000	189.0	25.1	58.3	582.4	117.6	282.7
240	0	302.6	42.6	88.8	806.4	185.9	435.5
	125	286.6	40.3	86.2	745.4	180.3	415.3
	500	247.8	37.5	81.5	691.6	142.2	364.6
	2000	215.3	29.7	70.3	623.3	136.6	342.0

Table XII : <u>Effect of iodoacetic acid (500 mg/L) on the release of the Water-Soluble, Formol and Non-Protein nitrogen in barley and malt.</u>

Autolysis Time (min.)	Inhibitor conc. mg/L*	BARLEY mg. per 100 g. WSN	BARLEY FN	BARLEY NPN	MALT mg. per 100 g. WSN	MALT NP	MALT NPN
15	0	118.8	12.3	27.8	436.8	79.5	190.4
	500	102.8	10.8	26.8	418.9	77.3	187.0
30	0	156.4	18.5	35.1	534.8	86.9	232.0
	500	141.6	15.7	33.1	506.8	86.3	220.0
60	0	201.6	22.4	46.4	616.0	119.8	288.6
	500	194.7	21.5	41.7	585.2	116.5	266.5
120	0	261.5	30.2	67.0	700.0	146.7	351.6
	500	247.2	28.5	60.9	606.3	136.6	338.0
240	0	302.6	42.6	88.8	806.4	185.9	435.5
	500	296.3	39.1	84.8	778.4	169.1	422.5

* The corresponding approximate mM concentration of 500 mg/L iodoacetic acid is 2.7.

WSN = Water-Soluble nitrogen

FN = Formol nitrogen

NPN = Non-Protein nitrogen

Table XIII : Effect of various concentrations of thioglycollic acid (NaSalt) on the release of the Water-Soluble, Formol and Non-Protein nitrogen in barley and malt.

Autolysis Time (min.)	Activator conc. mg/L*	B A R L E Y mg. per 100 g. WSN	FN	NPN	M A L T mg. per 100 g. WSN	FN	NPN
15	0	118.8	12.3	27.8	436.8	79.5	190.4
	125	131.6	13.4	23.1	403.2	80.6	178.7
	500	137.0	14.0	30.1	406.0	81.2	185.2
	2000	142.7	14.0	33.7	432.3	87.3	198.5
30	0	156.4	18.5	35.1	534.8	86.9	232.0
	125	163.6	19.0	42.3	519.6	93.0	232.7
	500	166.7	19.6	39.1	529.6	96.3	224.2
	2000	177.0	20.3	42.3	545.2	97.4	239.0
60	0	201.6	22.4	46.4	616.0	119.8	288.6
	125	216.7	23.5	51.7	610.4	119.8	271.7
	500	217.5	24.6	53.7	616.4	123.1	278.8
	2000	238.7	27.8	57.5	627.6	125.4	289.2

* The corresponding approximate mM concentrations of 125, 500 and 2,000 mg/L thioglycollic (Na Salt) are 1.1, 4.4, and 17.6 respectively.

WSN = Water-Soluble nitrogen

FN = Formol nitrogen

NPN = Non-Protein nitrogen

Table: XIII (Contd.)

120	0	261.5	30.2	67.0	700.0	146.7	351.6
	125	264.9	31.9	67.0	665.8	147.0	354.9
	500	279.8	35.3	67.6	666.4	149.1	366.2
	2000	290.1	36.7	71.6	728.0	152.3	380.2
240	0	302.6	42.6	88.8	806.4	185.9	435.5
	125	310.6	43.1	92.8	712.9	201.6	450.8
	500	336.9	47.3	95.5	800.1	219.2	459.7
	2000	342.6	50.9	99.5	816.4	229.3	474.7

Table XIV : Effect of pH on the Hb-ase activity of a freeze-dried extract of malt.

pH	Hb-ase Activities	
	Specific Activity*	Relative Activity**
3.0	0.54	87.0
3.8	0.62	100.0
4.0	0.48	77.4
4.5	0.39	62.9
5.0	0.30	48.4
5.5	0.26	42.0
6.0	0.09	14.5
7.0	0.15	24.5
8.0	0.07	11.3
8.5	0.06	9.7
9.0	0.06	9.7

*Specific activity is expressed as extinction at 280 mμ per hour per mg. protein.

** Activity relative to activity at pH 3.8, taken as 100.

Table XV : Effect of temperature (C°) on the rate of deactivation of Hb-ase of a freeze-dried extract of malt.

Temperature (c°)	Hb-ase Activities	
	Specific Activity*	Relative Activity**
35	0.46	100.0
40	0.46	100.0
45	0.30	65.0
50	0.24	56.2
60	0.10	21.7
70	0.08	17.4
80	0.06	13.0

*Specific activity is expressed as extinction at 280 mμ per hour per mg. protein.

** Activity relative to activity at 35 to 40°C taken as 100.

Table XVI : Effect of time of assay on the rate of hydrolysis of 1.5% Hb at pH 3.8 and 40°C and azo-glutenin at pH 5.0 and 40°C by malt enzyme(s).

Time of Assay (min.)	Specific Activity*	
	Hb-ase	Azo-glutenin-ase
10	0.06	
20	0.26	0.04**
30	0.36	0.08
60	0.62	0.14
90	0.80	0.18
120	0.88	0.22
180	1.08	

*Specific activities of Hb-ase and Azo-glutenin-ase are expressed as extinction units at 280 and 460 mμ respectively per mg. protein.

**Assay time 15 minutes.

Table XVII: Effect of enzyme concentration (mg. protein of freeze-dried malt extract) on the rate of hydrolysis of 1.5% Hb- at pH 3.8 and 40°C.

mg. protein in freeze-dried malt extract.	Hb-ase activity*
1	0.25
2	0.36
3	0.66
4	0.84
5	1.03

*Hb-ase activity is reported as extinction units at 280 mμ per hour per ml. of enzyme solution.

Table XVIII: Effect of substrate (Hb) concentration on its rate of hydrolysis by malt enzyme (s) at pH 3.8 and 40°C.

Substrate (Hb) conc. (%)	Hb-ase Activites	
	Specific Activity*	Relative Activity**
0.5	0.46	67.0
1.0	0.68	100.0
1.5	0.67	98.5
2.0	0.59	86.8
2.5	0.56	82.4

*Specific activity is expressed as extinction at 280 mμ per hour per mg. protein.

**Activity relative to activity at substrate concentration of 1.0% taken as 100.

Table XIX: Effect of enzyme concentration (mg. protein in freeze-dried malt extract) on the rate of hydrolysis of BAPA and BAEE.

Mg. protein in the freeze-dried malt extract.	Enzyme Activities*	
	BAPA-ase	BAEE-ase
1	0.21	0.31
2	0.30	0.43
3	0.35	0.47
4	0.38	0.56
5		0.59

*BAPA-ase and BAEE-ase activities are reported as extinction units at 410 and 253 mμ respectively per hour per ml. of enzyme solution.

Table XX : Effect of time of assay on the rate of hydrolysis of BAPA by malt enzyme and commercial trypsin.

Assay Time (min)	Enzyme Activities*	
	Malt enzyme	Commercial trypsin
5	-	0.05
10	-	0.11
15	0.04	0.15
20	-	0.19
30	0.09	0.28
45	0.14	-
60	0.19	0.54
90	0.27	-
120	0.33	-

*Activity of malt enzyme is reported as extinction at 410 mμ per mg.protein and that of commercial trypsin as extinction at 410 mμ per ml. of water containing 100 μg of enzyme.

Table XXI : Fractionation of proteases from malt by ammonium sulphate precipitation.

Ammonium Sulphate Saturation (%)	Composition of precipitate as percent of total in the extract			
	Protein (mg)	Hb-ase activity	BAPA-ase activity	BAA-ase activity
0 - 20	5.3	0.0	1.8	8.2
20 - 40	18.7	1.4	13.3	10.6
40 - 60	31.6	33.8	46.4	19.4
60 - 80	11.8	25.7	10.4	10.6
80 - 100	1.6	9.4	0.8	3.5
Mother liquor	5.0	2.7	0.8	*
Total Recovery (%)	74.0	73.0	73.5	52.3

The origianal extract 140 ml. from 100g. malt contained, before fractionation with ammonium sulphate, 616 mg. of protein and had 187.6, 84.0 and 833.0 units of Hb-ase, BAPA-ase and BAA-ase activities respectively. Hb-ase and BAPA-ase activities are reported as extinction units at 280 and 410 mμ respectively per ml. (of the fraction) per hour. BAA-ase activity is reported as μg. of ammonia liberated per ml. (of the fraction) per hour.

* Not detected.

Table XXII: **Effect of various concentrations of potassium bromate on the Hb-ase and BAPA-ase activities of freeze-dried extract of malt precipitated at 20 to 80% ammonium sulphate saturation.**

Potassium Bromate Conc. (M)	Hb-ase Activity		BAPA-ase Activity	
	Specific Activity	Relative Activity	Specific Activity	Relative Activity
0	0.66	100.0	0.13	100
1×10^{-4}	0.69	104.5		
1×10^{-3}	0.67	101.5	0.12	92.3
1×10^{-2}	0.44	66.6	0.13	100.0
1×10^{-1}	0.27	40.9		

Specific activities of Hb-ase and BAPA-ase are reported as extinction units at 280 and 410 mμ respectively per hour per mg. protein.

Table XXIII: **Effect of various concentrations of iodoacetamide on the Hb-ase and BAPA-ase activities of freeze-dried extract of malt precipitated at 20 to 80% ammonium sulphate saturation.**

Iodoacetamide Conc. (M)	Hb-ase Activity		BAPA-ase Activity	
	Specific Activity	Relative Activity	Specific Activity	Relative Activity
0	0.74	100.0	0.14	100.0
1×10^{-4}	0.84	113.5		
1×10^{-3}	0.82	110.8	0.17	121.0
1×10^{-2}	0.84	113.5	0.16	114.0
1×10^{-1}			0.13	93.0

Specific activities of Hb-ase and BAPA-ase are reported as extinction units at 280 and 410 mμ respectively per hour per mg. protein.

Table XXIV: **Effect of various concentrations of N-Ethylmaleimide on the Hb-ase and BAPA-ase activities of freeze-dried extract of malt precipitated at 20 to 80% ammonium sulphate saturation.**

N-Ethylmaleimide	Hb-ase Activity		BAPA-ase Activity	
Conc. (M)	Specific Activity	Relative Activity	Specific Activity	Relative Activity
0	0.66	100.0	0.15	100.0
1×10^{-5}	0.69	104.5		
1×10^{-4}	0.66	100.0	0.16	106.6
1×10^{-3}	0.70	106.0	0.13	86.6
1×10^{-2}	0.54	82.0	0.07	46.6

Specific activities of Hb-ase and BAPA-ase are reported as extinction units at 280 and 410 mμ respectively per hour per mg. protein.

Table XXV: Effect of various concentrations of phenylmercuryacetate on the Hb-ase activity of freeze-dried extract of malt precipitated at 20 to 80% ammonium sulphate saturation.

Phenylmercuryacetate Conc. (M)	Hb-ase Activity	
	Specific Activity	Relative Activity
0	0.72	100.0
1×10^{-6}	0.70	97.2
1×10^{-5}	0.64	88.8
1×10^{-4}	0.60	83.3
1×10^{-3}	0.52	72.2
1×10^{-2}	0.17	23.6

Specific activity of Hb-ase is reported as extinction units at 280 mμ per hour per mg. protein.

Table XXVI: Effect of various concentrations of p-chloromercuriphenylsulphonic acid on the Hb-ase activity of freeze-dried extract of malt precipitated at 20 to 80% ammonium sulphate saturation.

p-Chloromercuri phenylsulphonic acid Conc. (M)	Hb-ase Activity	
	Specific Activity	Relative Activity
0	0.65	100.0
1×10^{-6}	0.67	103.0
1×10^{-5}	0.70	107.7
1×10^{-4}	0.63	97.0
1×10^{-3}	0.54	83.0
1×10^{-2}	0.49	75.4

Specific activity is reported as extinction units at 280 mμ per hour per mg. protein.

Table XXVII: Effect of various concentrations of p-hydroxychloromercuribenzoate on the BAPA-ase activity of freeze-dried extract of malt precipitated at 20 to 80% ammonium sulphate saturation.

p-Hydroxychloro mercuribenzoate Conc. (M)	BAPA-ase Activity	
	Specific Activity	Relative Activity
0	0.14	100.0
1×10^{-5}	0.12	85.7
1×10^{-4}	0.07	50.0
1×10^{-3}	0.05	35.7

Specific activity is reported as extinction units at 410 mμ per hour per mg. protein.

Table XXVIII. Effect of various concentrations of ethylenediaminetetraacetic acid on the Hb-ase and BAPA-ase activities of freeze-dried extract of malt precipitated at 20 to 80% ammonium sulphate saturation.

Ethylenediaminetetraacetic acid	Hb-ase Activity		BAPA-ase Activity	
Conc. (M)	Specific Activity	Relative Activity	Specific Activity	Relative Activity
0	0.54	100.0	0.11	100.0
1×10^{-3}	0.59	109.2	0.13	118.1
1×10^{-2}	0.71	131.5	0.14	127.2
1×10^{-1}	0.69	127.7	0.14	127.2

Specific activities of Hb-ase and BAPA-ase are reported as extinction units at 280 and 410 mμ respectively per hour per mg. protein.

Table XXIX: **Effect of various concentrations of phenylmethylsulphonylfluoride on the BAPA-ase activity of freeze-dried extract of malt precipitated at 20 to 80% ammonium sulphate saturation and commercial trypsin.**

Phenylmethylsulphonyl fluoride	BAPA-ase Activity			
	From Malt		Commercial Trypsin	
Conc. (M)	Specific Activity	Relative Activity	Activity	Relative Activity
0	0.24	100.0	0.29	100.0
1×10^{-5}	0.24	100.0	0.28	96.5
1×10^{-4}	0.25	104.2	0.26	89.6
1×10^{-3}	0.25	104.2	0.21	72.4

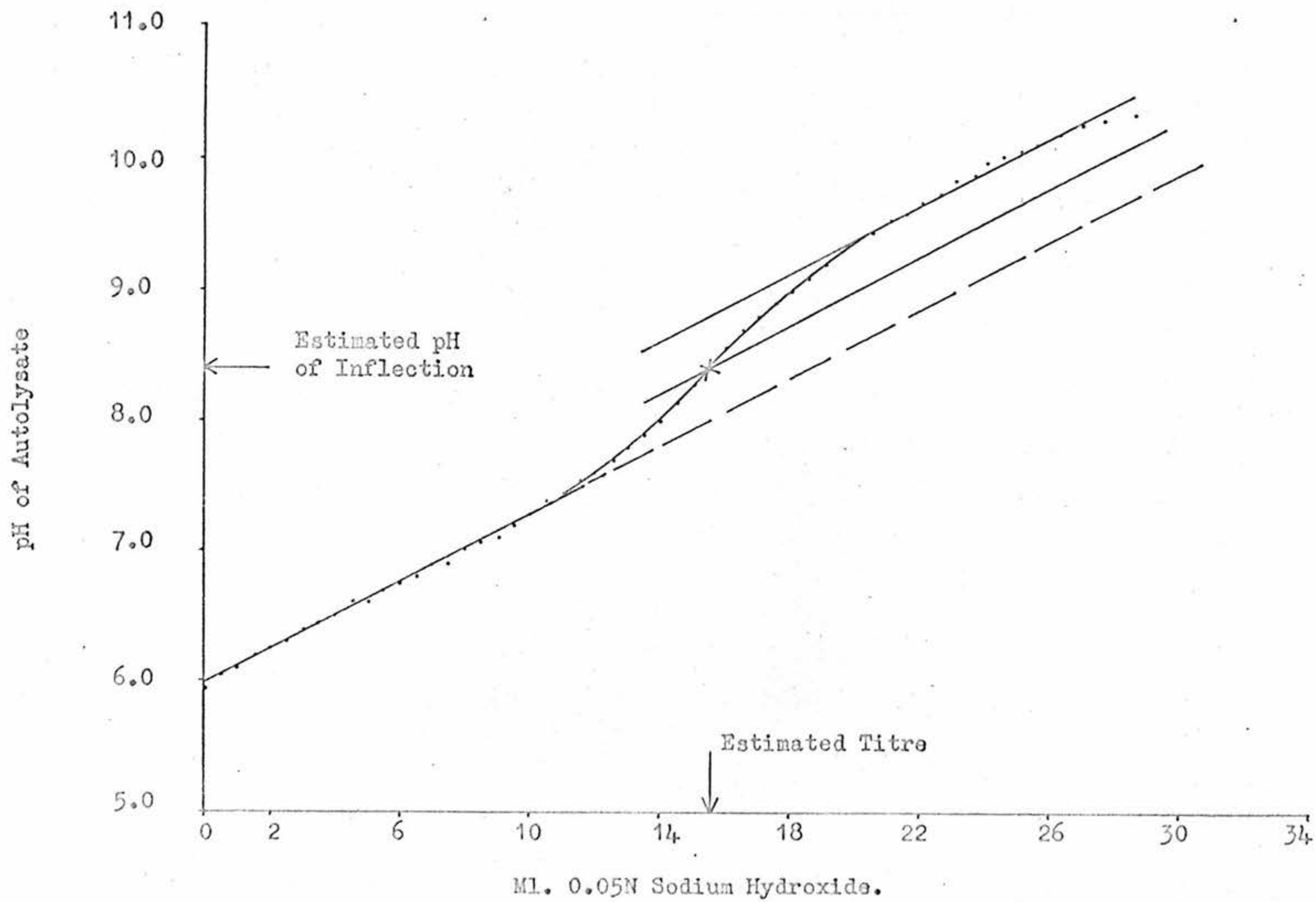
Specific activity of malt BAPA-ase is reported as extinction units at 410 mμ per hour per mg. protein. Activity of commercial trypsin is reported as extinction units at 410 mμ per hour per ml. of water containing 25 μg. of commercial trypsin.

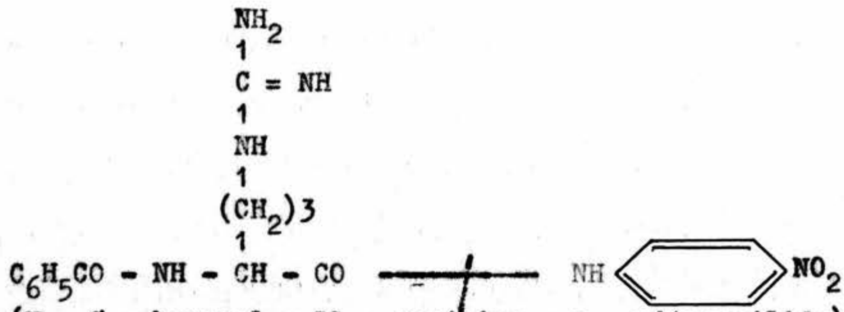
Table XXX : Development of Hb-ase and BAPA-ase in Germinating barley.

Stage of Germination	Protein and Enzyme Activities*		
	Protein (mg)	Hb-ase Activity	BAPA-ase Activity
Ungerminated barley	3.6	0.45	0.08
Steeped barley	3.6	0.65	1.00
1 day	3.2	0.65	0.95
2 days	3.0	0.65	0.95
3 days	3.6	0.80	1.45
4 days	4.6	1.25	1.90
5 days	5.4	1.40	2.55
7 days	4.7	1.40	2.55
9 days	4.7	1.35	2.45

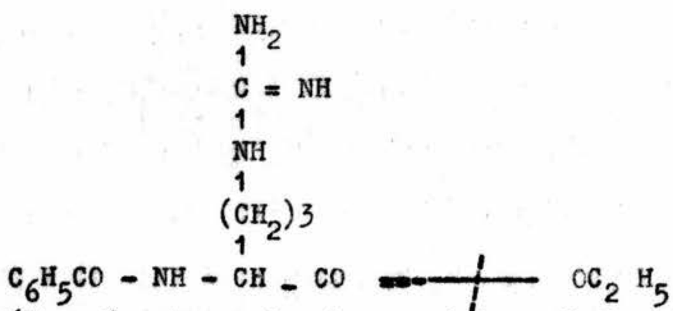
*Hb-ase and BAPA-ase activities are expressed as extinction units at 280 and 410 mμ respectively per hour per g. of barley. Protein concentration is expressed as mg/ml. of the final extract volume (50 ml.) from 10 g. of barley. All results reported are mean of three separate determinations.

Fig. 1: Titration curve of a 240 minutes autolysate (50 ml.) obtained from Proctor barley (25 g.) of the 1963 harvest with 0.05 N sodium hydroxide.

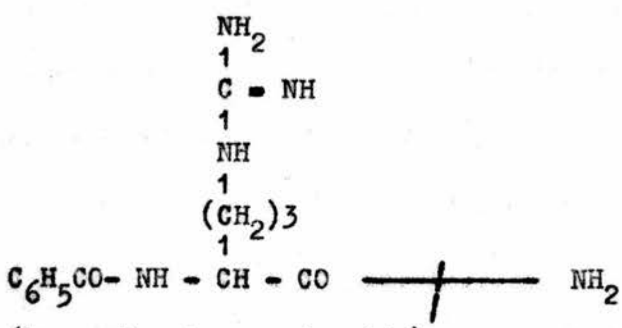


1. 

(N - α - benzoyl - DL - arginine - p - nitroanilide)

2. 

(N - α - benzoyl - L - arginine ethyl ester)

3. 

(benzoyl - L - arginamide)

Fig. 2: Synthetic substrates used for the assay of "trypsin-like" enzyme(s) of a freeze-dried extract of malt.
The dotted lines indicate the points of hydrolysis of the substrates.

Fig. 3: The release of the Water-Soluble, Formol and Non-Protein nitrogen in six barley varieties autolysed for 15, 30, 60, 120 and 240 minutes.

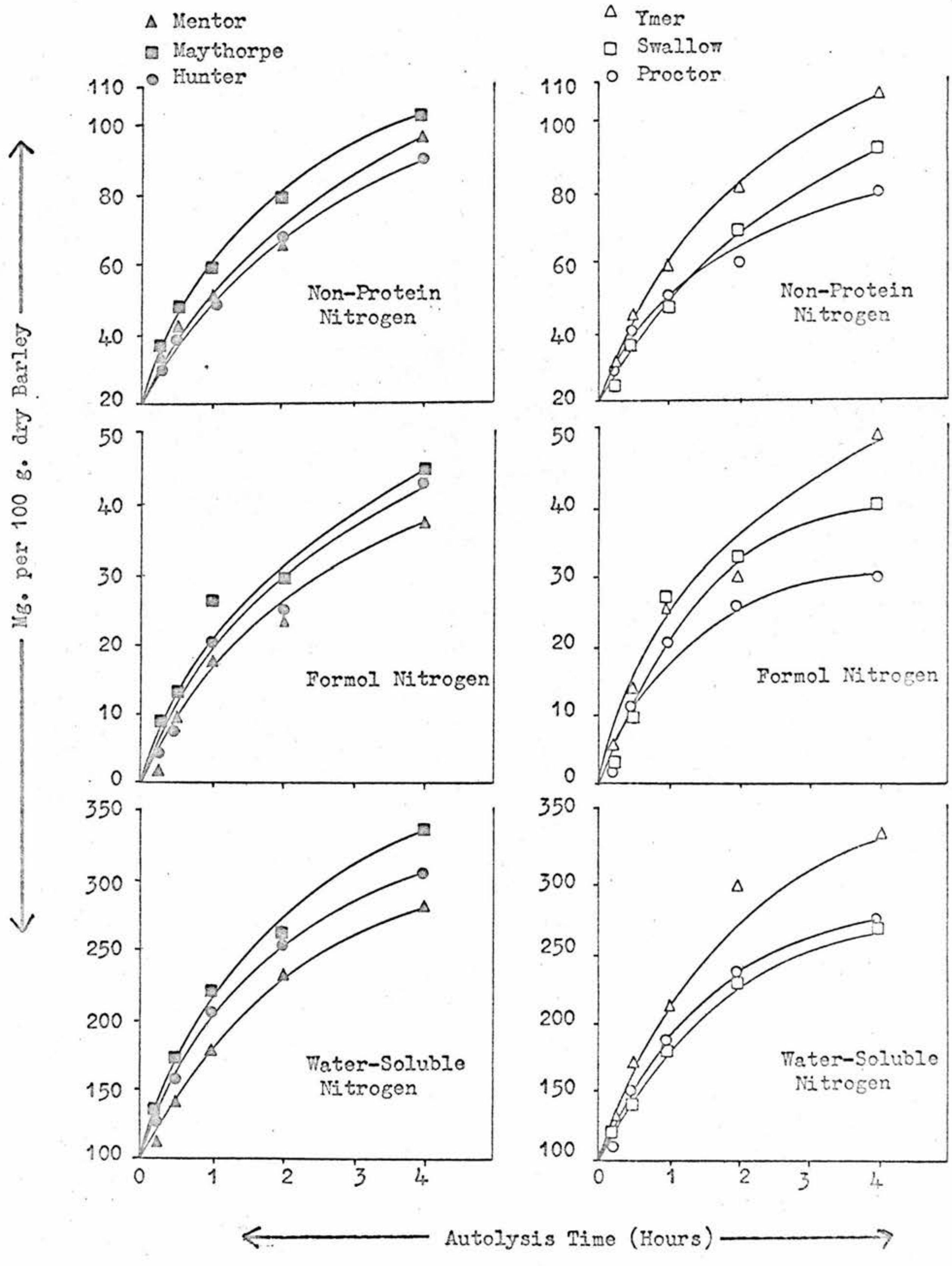


Fig. 4: The Water-Soluble nitrogen expressed as percent of the Total nitrogen of the barley and the Formol and Non-Protein nitrogen expressed as percentages of the Water-Soluble nitrogen of six barley varieties autolysed for 15, 30, 60, 120 and 240 minutes.

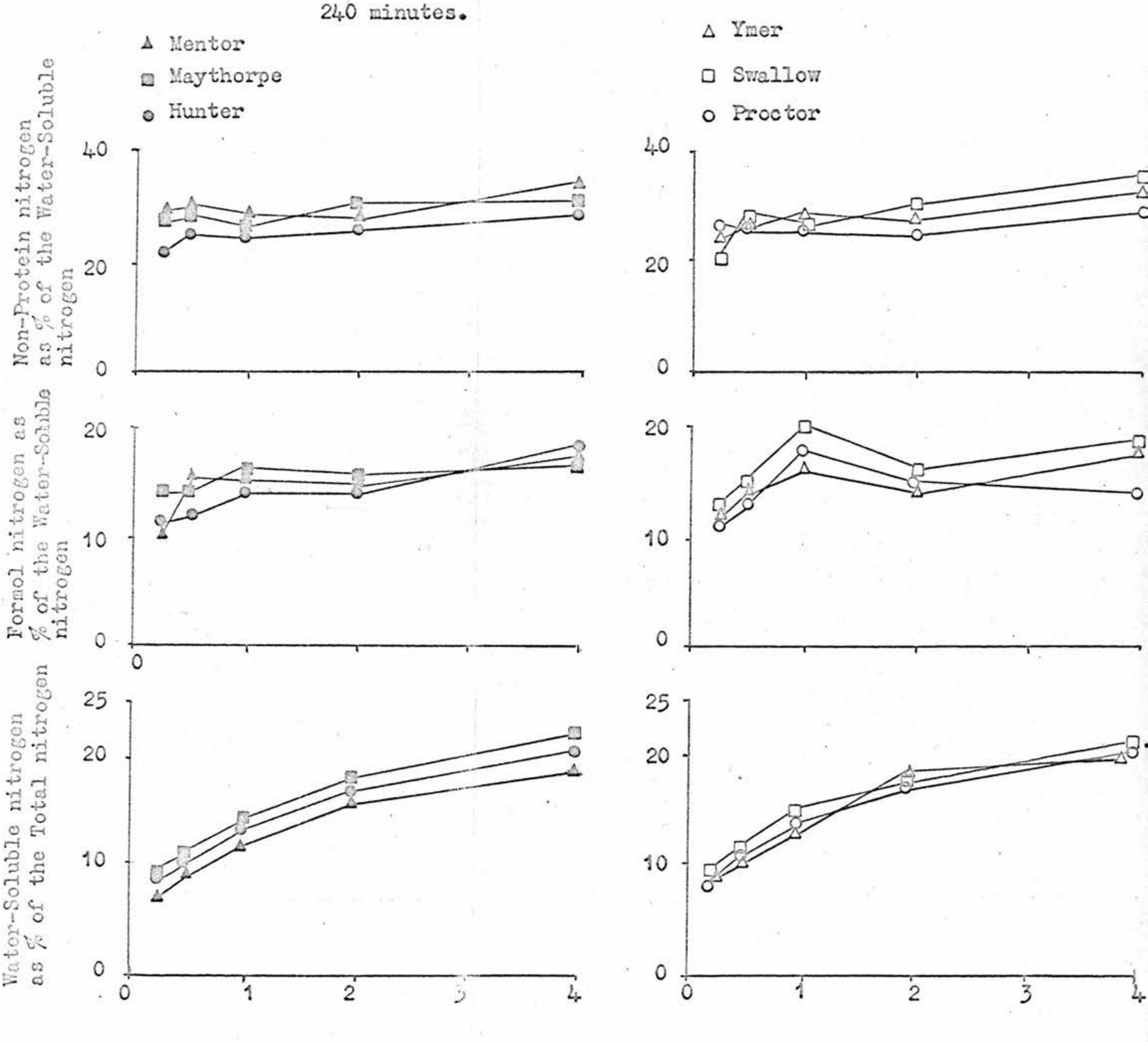


Fig. 5: Effect of pH on the Hb-ase activity of a freeze-dried extract of malt.

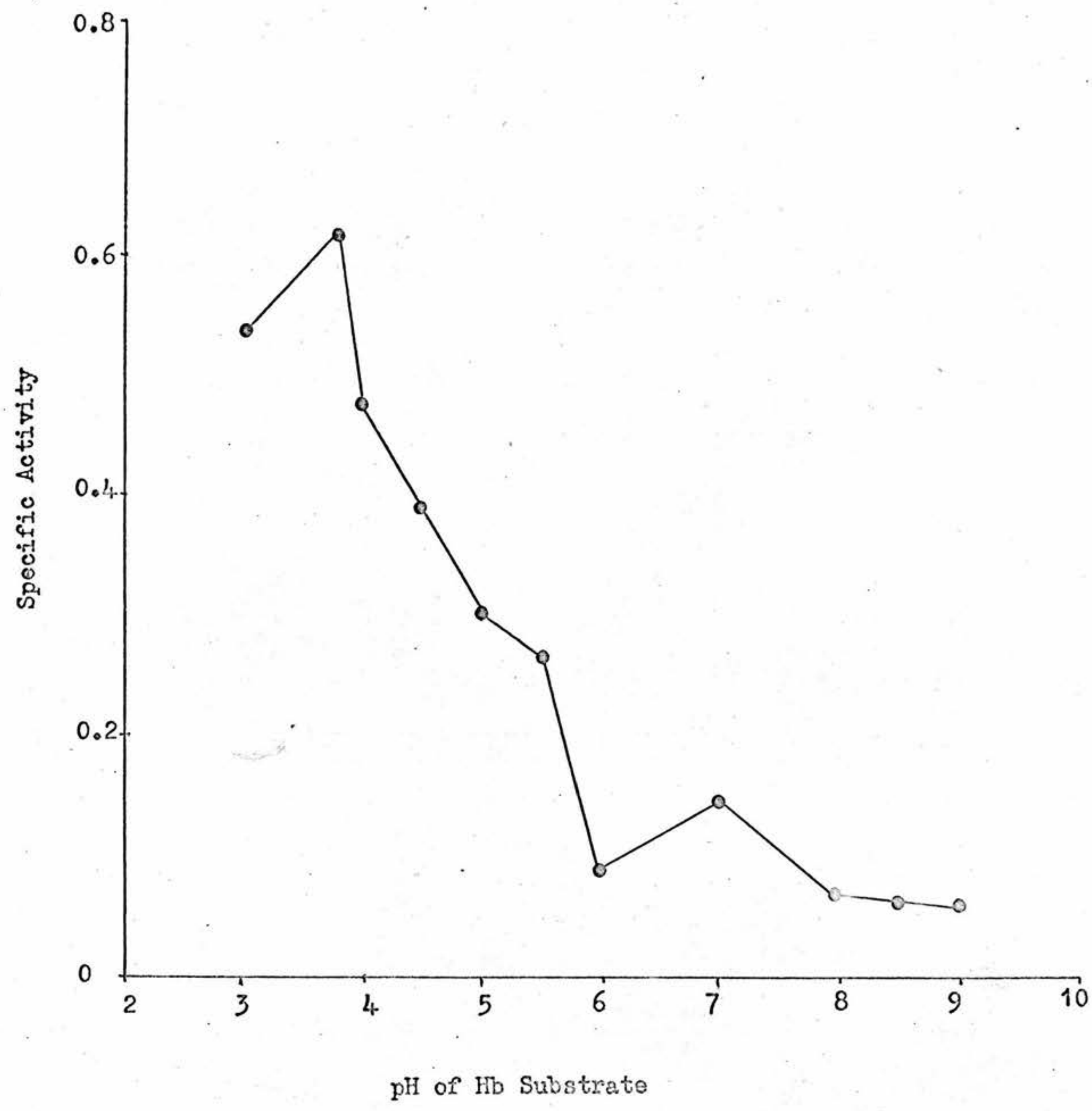


Fig. 6: Effect of temperature (C^{o}) on the rate of deactivation of Hb-ase of a freeze-dried extract of malt.

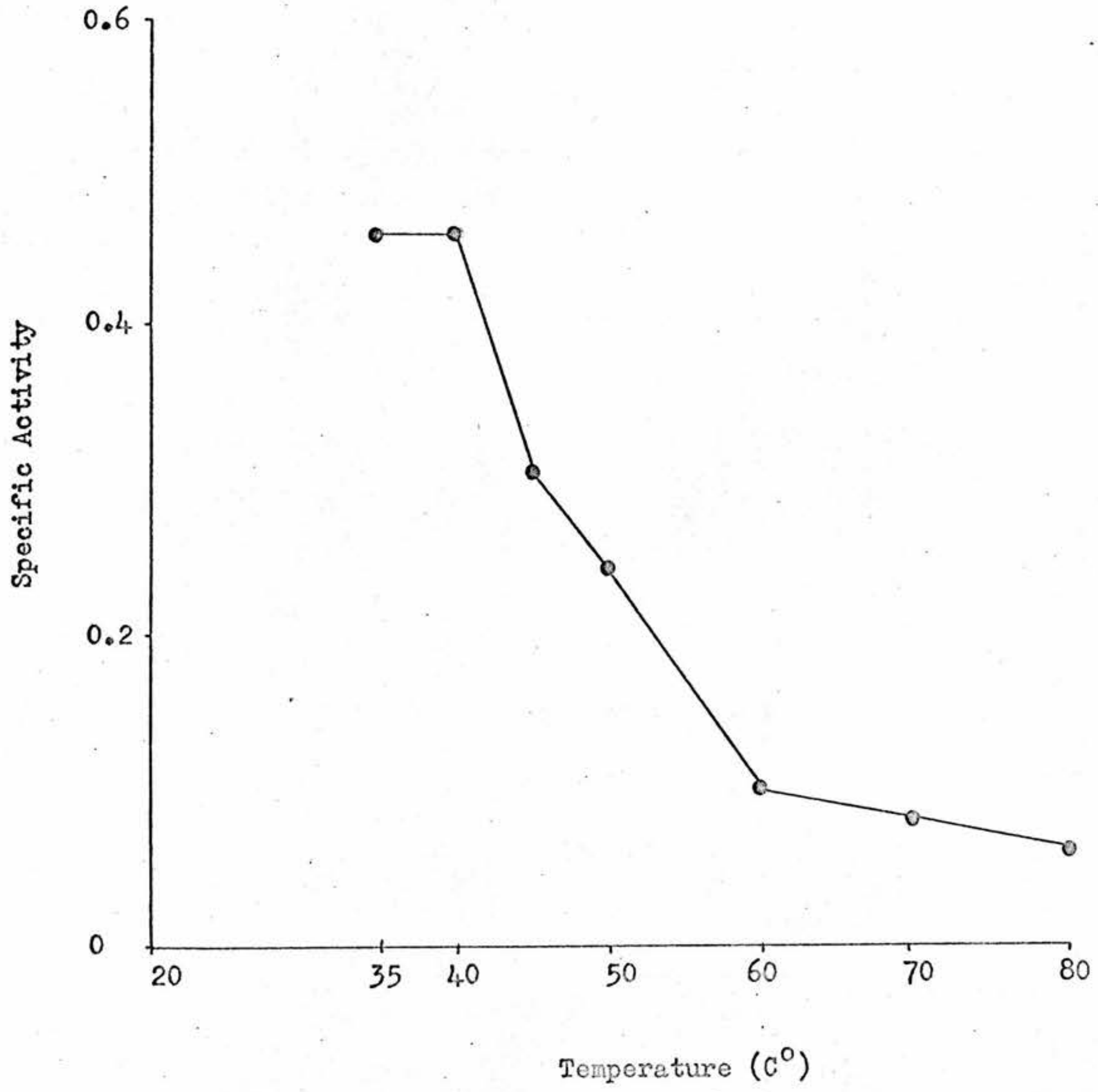


Fig. 7: Effect of time of assay on the rate of hydrolysis of 1.5% Hb at pH 3.8 and 40°C and 1% Azo-glutenin at pH 5.0 and 40°C by enzyme(s) of a freeze-dried extract of malt.

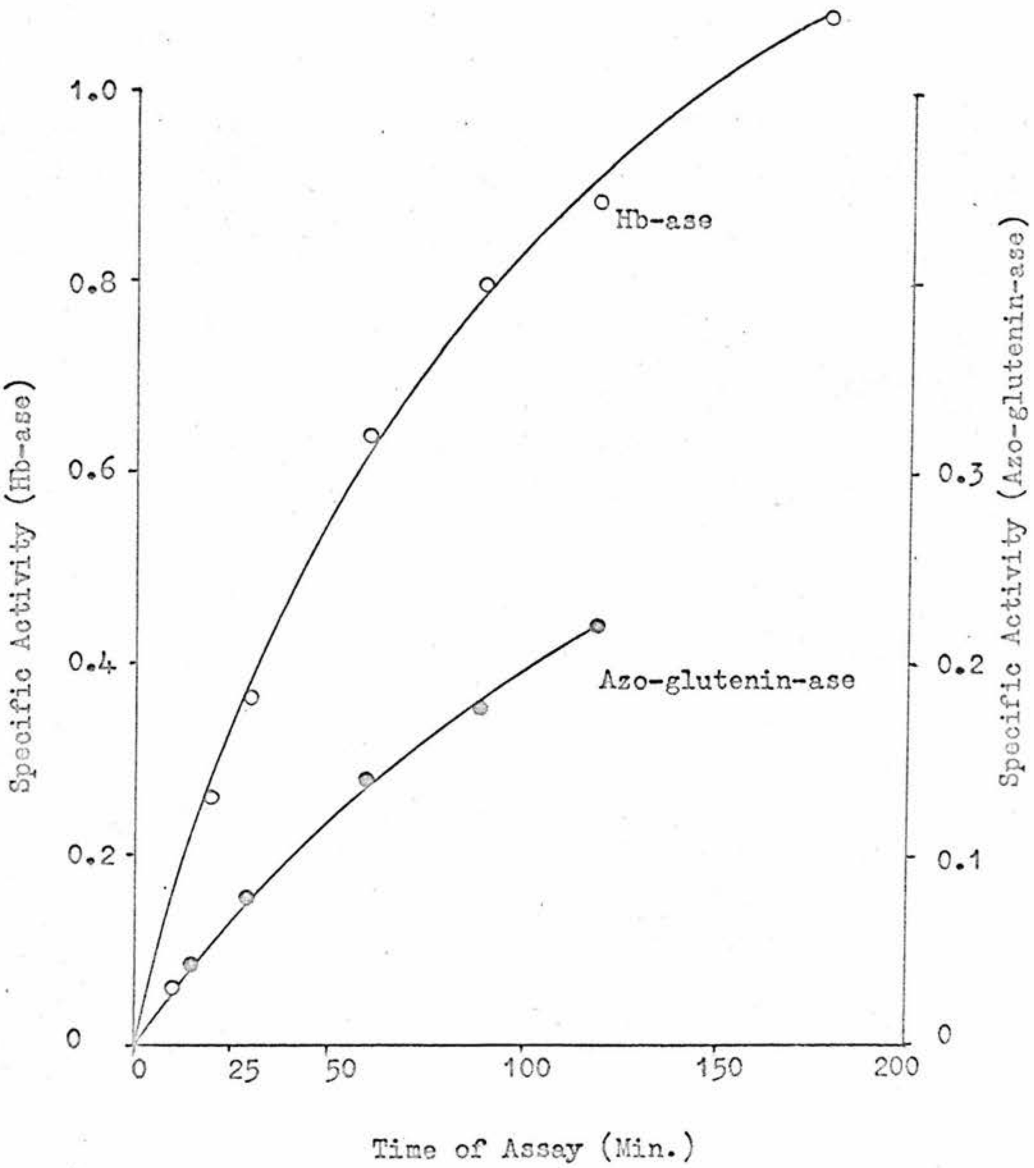


Fig. 8: Effect of enzyme concentration on the rate of hydrolysis of 1.5% Hb at pH 3.8 and 40°C.

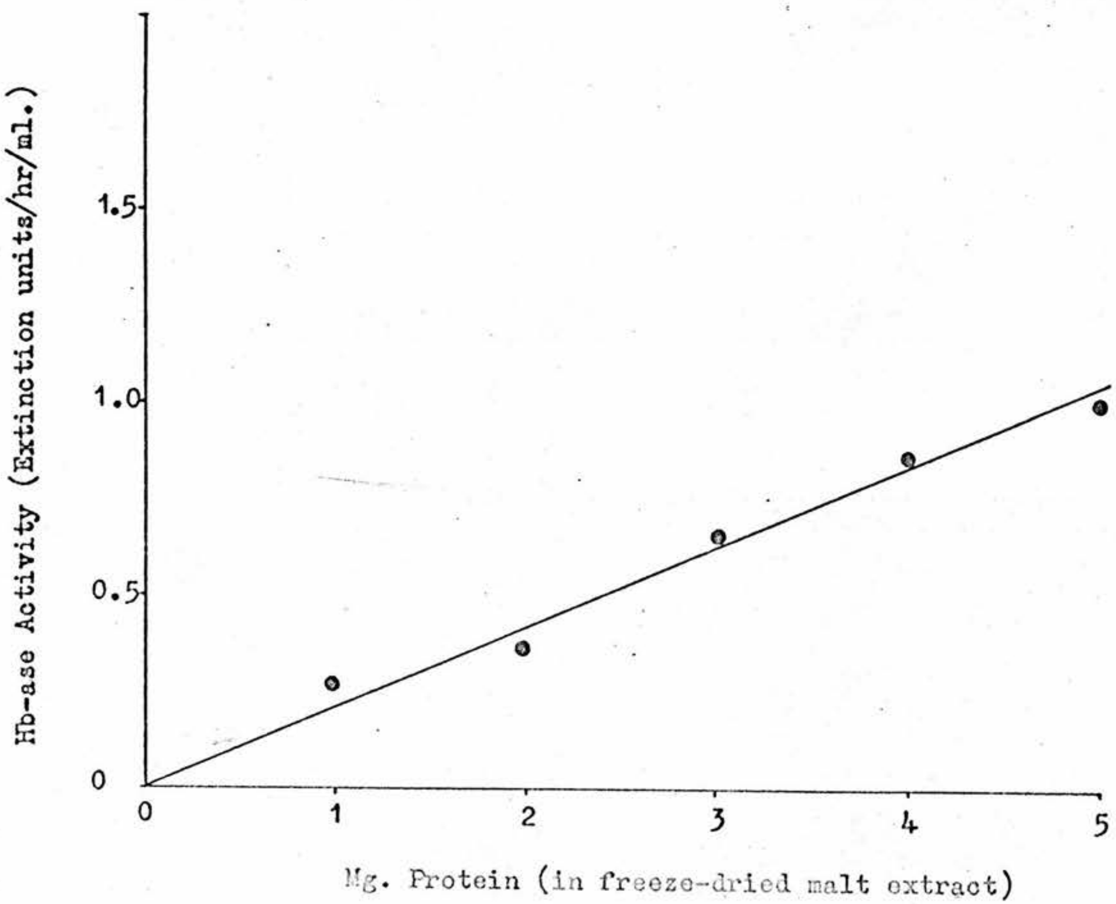


Fig. 9: Effect of substrate (Hb) concentration on its rate of hydrolysis by malt enzyme at pH 3.8 and 40°C.

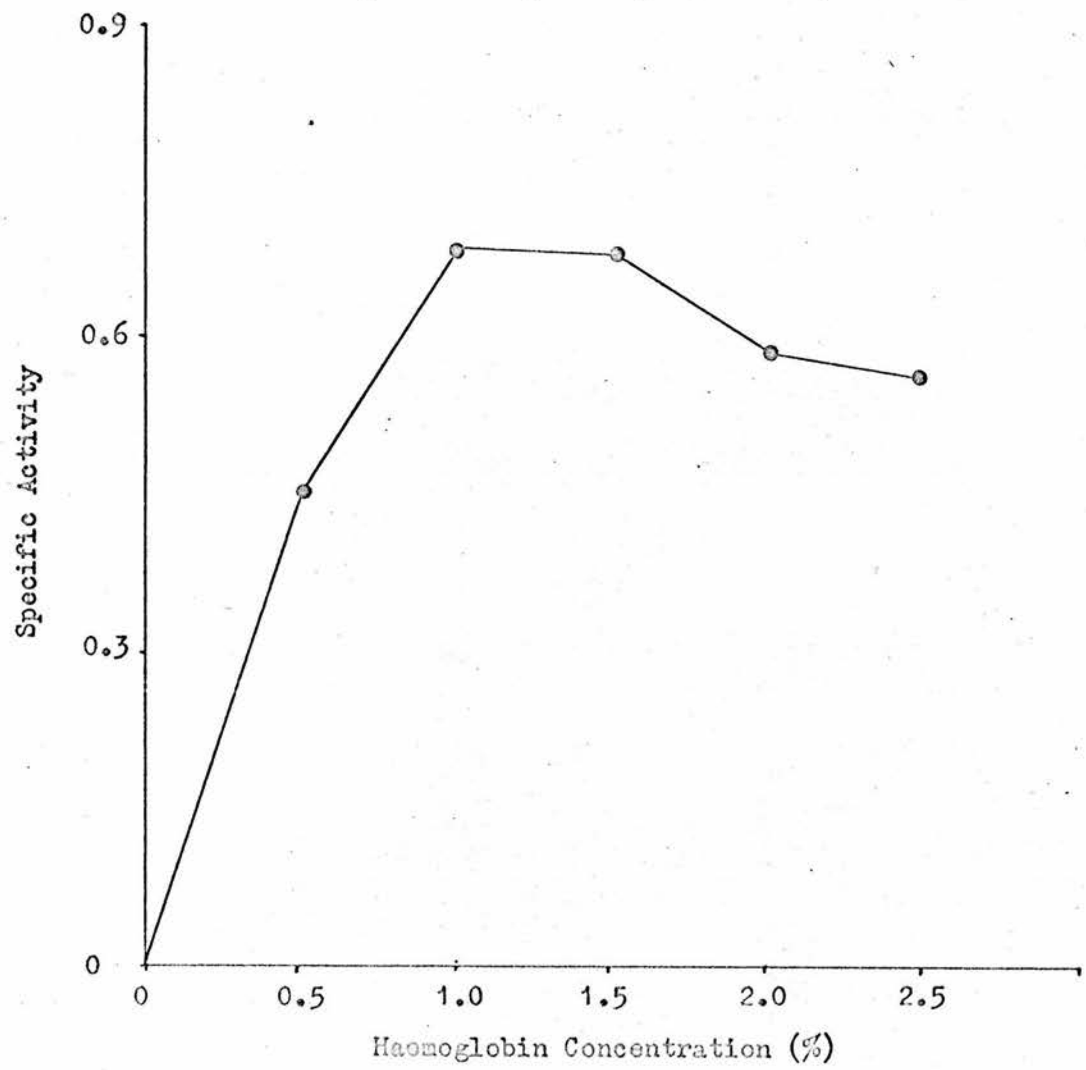


Fig. 10: Effect of time of assay on the rate of hydrolysis of α- benzoyl - L - arginine - p- nitroanilide (BAPA) by malt enzyme and commercial trypsin.

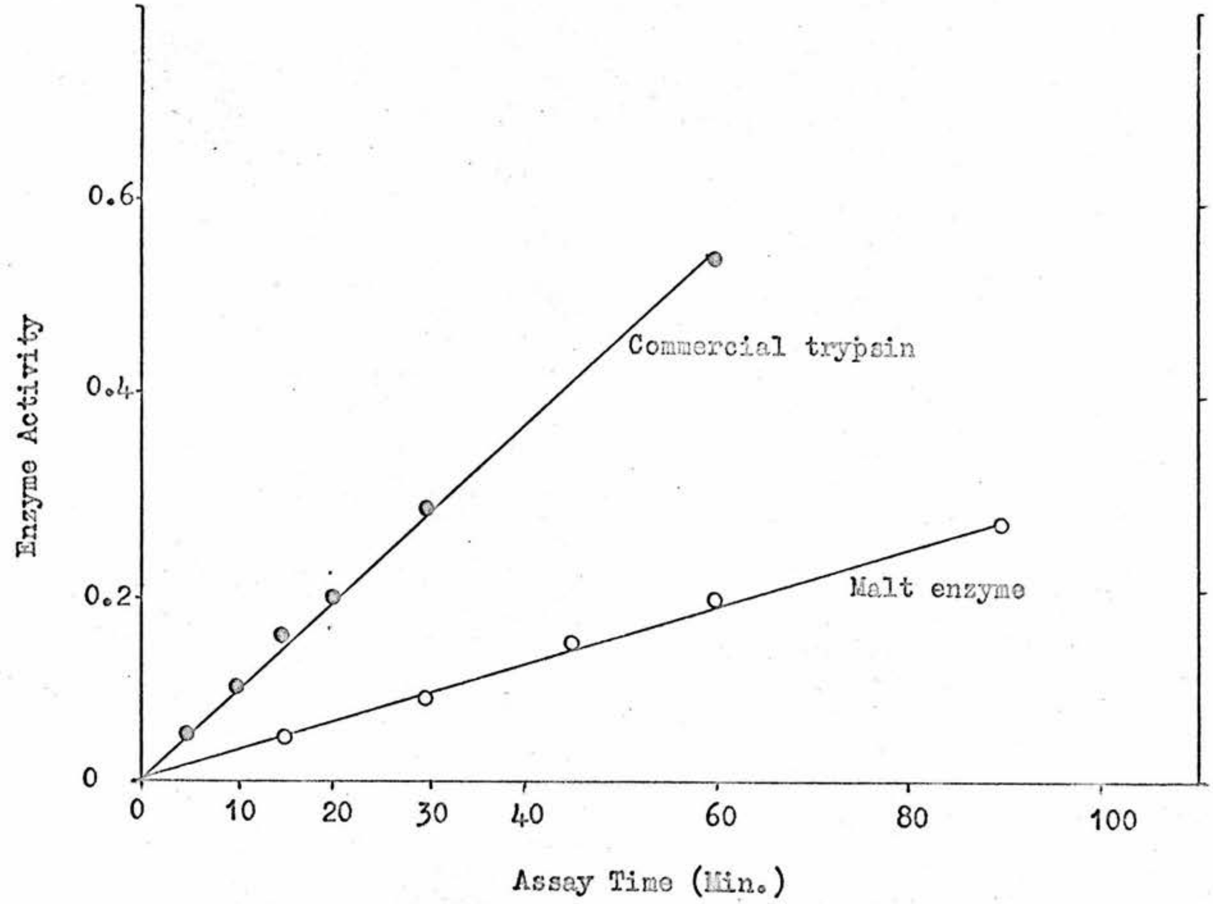


Fig. 11: Exclusion chromatography of 125 mg. of the freeze-dried extract of malt on Sephadex G-75 eluted ~~units~~ with 0.05M sodium acetate buffer pH 5.0. The Hb-ase activity is expressed as extinction at 280 mμ per hour per ml. of the fraction.

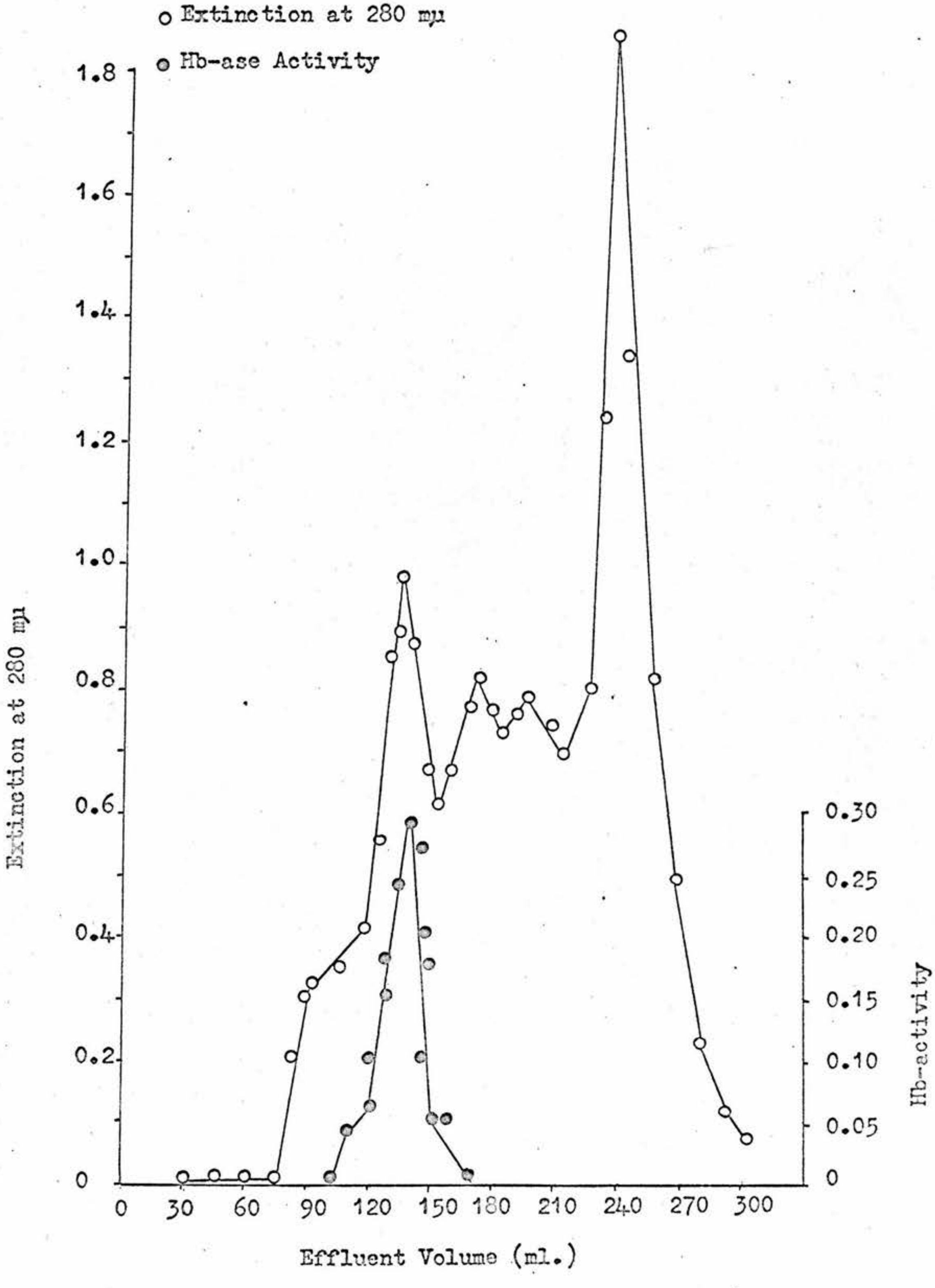


Fig. 12: Step-wise elution of 75 mg. of the freeze-dried extract of malt on DEAE-Cellulose . The Hb-ase activity is expressed as extinction at 280 mμ per hour per ml. of the fraction.

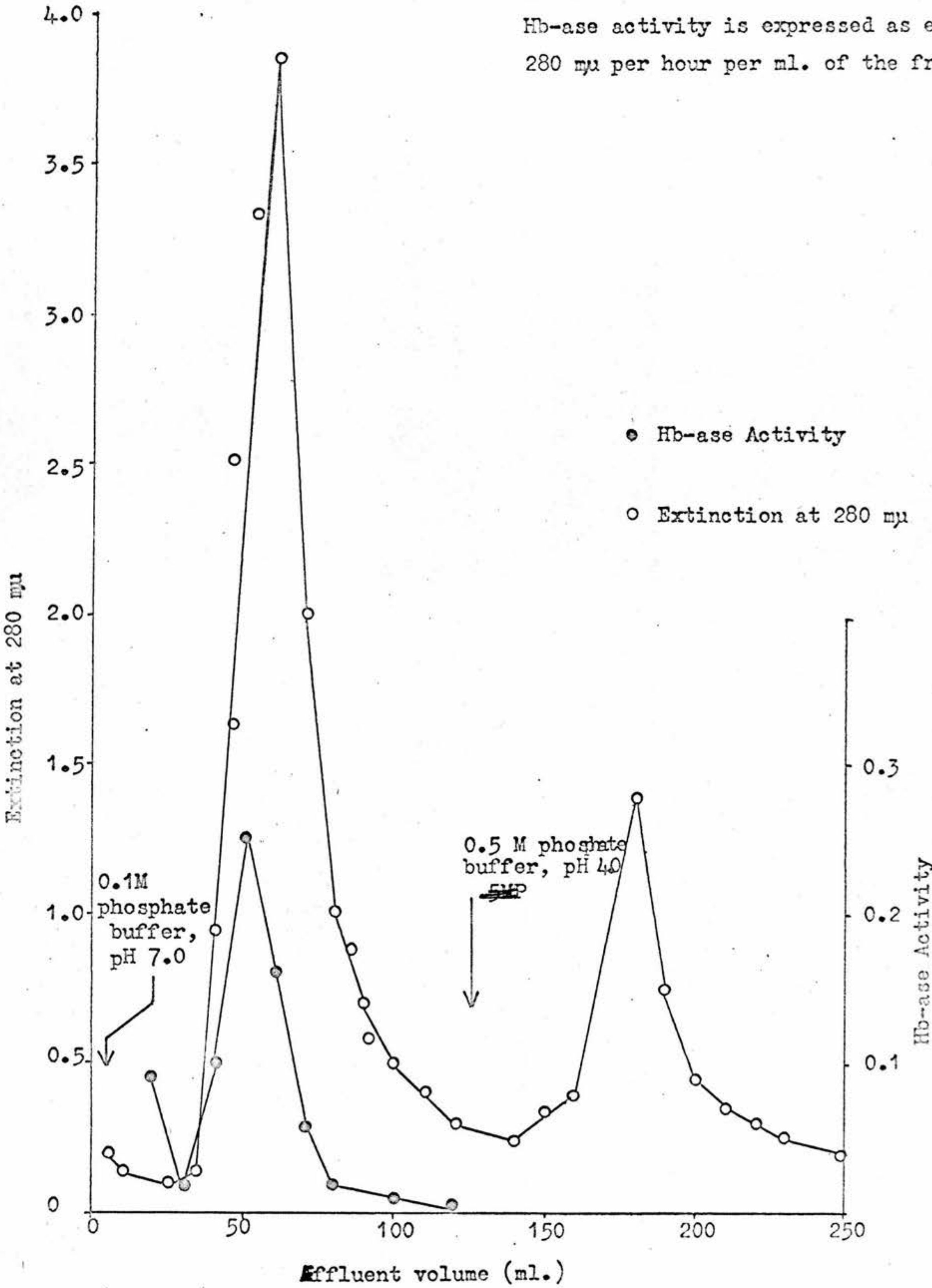


Fig. 13: Exclusion chromatography of 65 mg. of the 20 to 80% ammonium sulphate precipitated fraction of malt on Sephadex G-100 with 0.05M phosphate buffer, pH 5.8 containing 2.5% (w/v) sodium chloride. The Hb-ase and BAPA-ase activities are reported as extinction units at 280 and 410 mμ respectively per hour per ml. of the fractions.

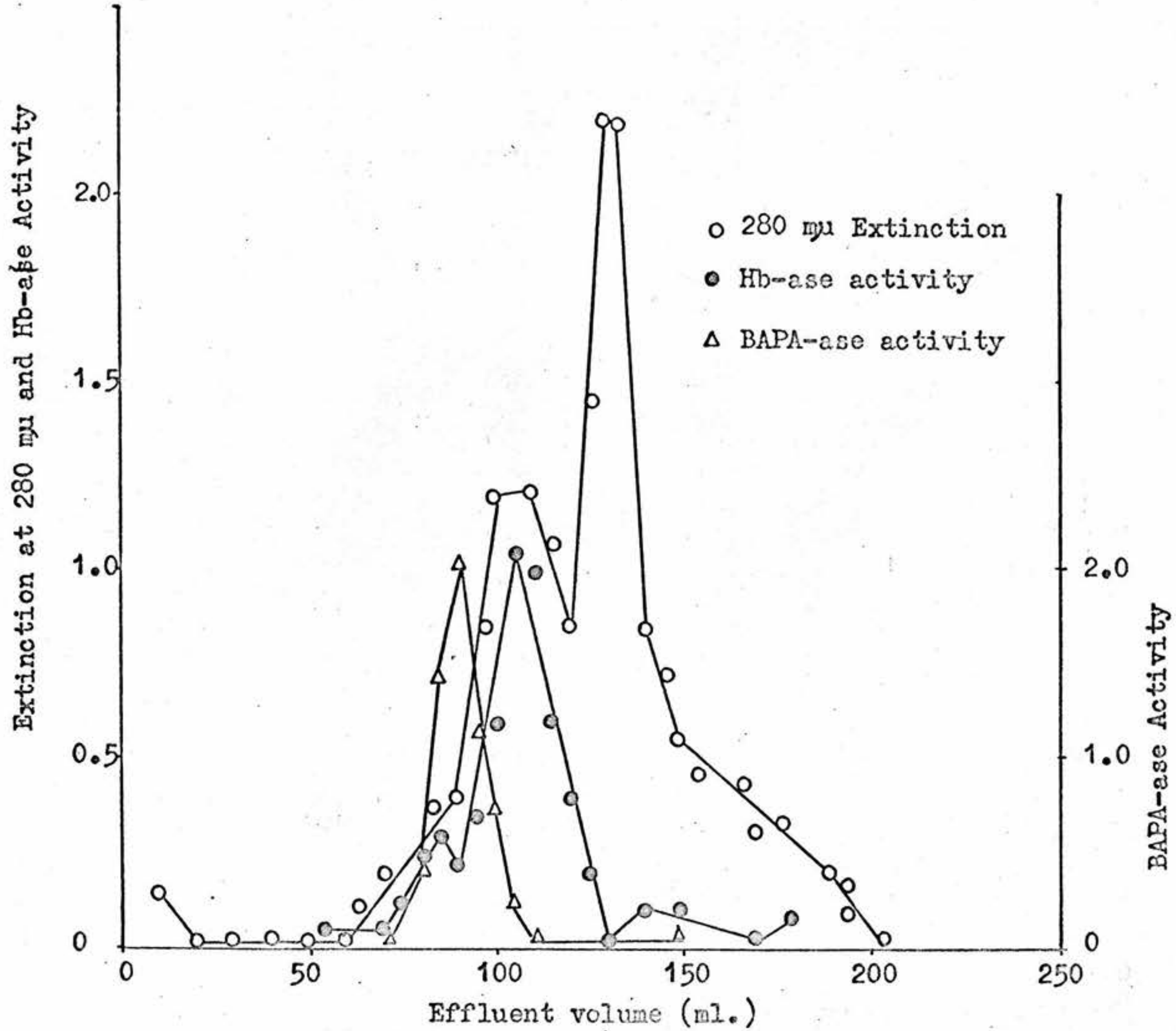
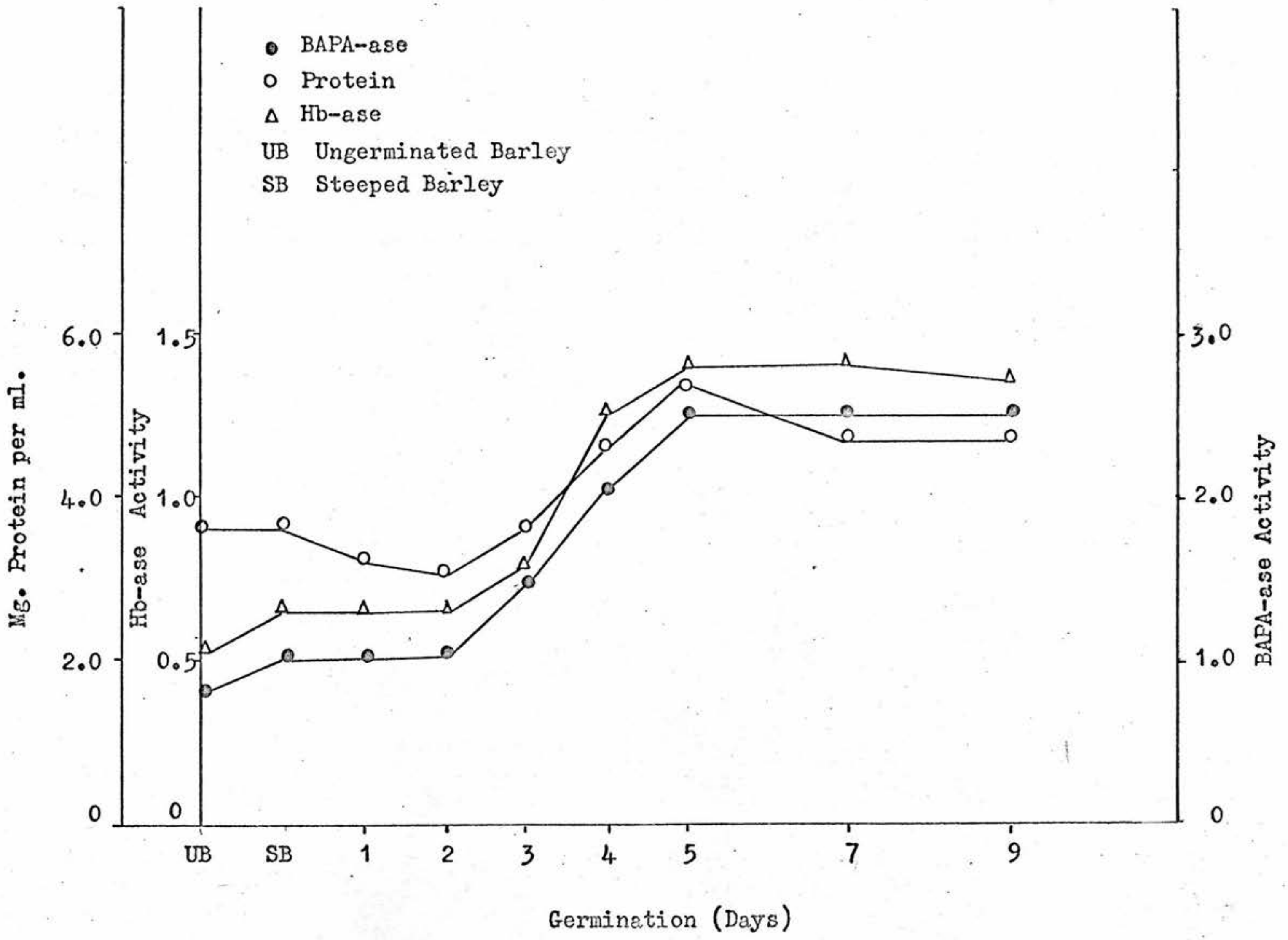


Fig. 14: The development of Hb-ase and BAPA-ase in germinating barley.



DISCUSSION

(A) Autolysis of Barley and Malt.

The primary aim of the work reported in this section of the thesis was to distinguish, if possible, six malting barley varieties of different protein content, on the basis of their proteolytic activity. The autolysis technique described by Preece and Aitken (1953) was employed to determine the proteolytic activity of the grain, which was measured by the release of the Water-Soluble, Formol and Non-Protein nitrogen. The autolysis technique offers the advantage over assay methods which employ foreign proteins as substrates in that it relies on the hydrolysis of the native proteins of barley or malt itself.

The formol titration method has been commonly employed for the estimation of non-protein nitrogenous substrates released by enzymic action. However, this method of measuring proteolysis is imprecise and arbitrary, the major difficulty being the determination of the end point to which the titration should be carried out. Because of the imprecision of this method, in the present study Formol nitrogen was estimated in the autolysates in conjunction with the Water-Soluble nitrogen and Lundin's fraction C, which measures low-molecular weight peptide nitrogen not precipitated by phosphomolybdic acid used as the protein precipitant.

Comparison of Proteolytic Activity of the Barley Varieties.

The differences in the production of the Water-Soluble, Formol and Non-Protein nitrogen in the varieties were not sufficiently large to suggest any definite varietal trends (Table

VIII and Fig. 3). The similarity in the proteolytic activity of the varieties became even more obvious when the Water-Soluble nitrogen was expressed as percent of the Total nitrogen and the Formol and Non-Protein nitrogen were expressed as percentages of the Water-Soluble nitrogen (Table VIII and Fig. 4). The evidence obtained, however, indicated that the proteolytic activity of the varieties varied with the nitrogen content of the grain, a high protein content being associated with a high proteolytic activity. A similar finding has also been reported by Sandegren (1951). From the point of view of revealing a new analytical method for assessing barley characteristics under routine conditions, the present results may be considered negative. However, it may be argued that such a pattern of results was obtained because the varieties compared were all of good malting quality. Comparison of these varieties with varieties of relatively poor malting quality might have indicated some discernible trends in the proteolytic activity of the grain. Such an approach might be attempted in a future study to obtain some indication of the proteolytic activity of newer barley varieties which may be developed for malting. If promising, such a routine technique could then be employed, in combination with other analyses in judging the suitability of barley varieties for brewing purposes without malting. The present methods of barley analysis give little information on the brewing quality of the barley variety, and, according to Sandegren (1951), results of malt analysis may be unreliable owing to variations introduced during the malting process.

<u>Effect of Inhibitors and an Activator on Proteolysis in Barley and Malt.</u>

The production of the Water-Soluble, Formol and

Non-Protein nitrogen both in barley and malt was inhibited by phenylmercuryacetate and iodoacetic acid and activated, though slightly, by the reducing agent thioglycollic acid (Tables XI, XII and XIII). Potassium bromate did not cause any inhibition of the nitrogen fractions estimated (Table X). Inhibition of the proteolytic activity of barley by potassium bromate (500 p.p.m. added to steep-water) has been reported by Macey and Stowell (1957), though under different experimental conditions than those employed in the present study. These results suggested that - SH enzymes were present in the autolysates of barley and malt. However, in a system such as an autolysate, the degree of activation or inhibition achieved will depend on the penetration of the solvent containing the compounds to the actual site of the enzyme. Furthermore, there are the possibilities that the inhibitors or activators are used up in attacking S - S or - SH bonds belonging to general protein material rather than at the active centre of the enzyme or that they are simply adsorbed to the grain materials. It is probably because of some or all these reasons that the activation or inhibition of proteolysis was small or negligible in relation to the concentration, particularly at the highest level, of the reagents employed.

(B) Fractionation of the Proteolytic Enzymes of the Malt.

Modern techniques of enzyme fractionation and purification have been little employed in the study of barley and malt proteolytic enzymes. The lack of avilability of sensitive and reproducible substrates, the presence of low quantities of proteolytic enzymes in the cereal grain, particularly in the ungerminated grain, and the low solubility of these enzymes in common enzyme extractants (Kringstad and Kilhovd, 1957; McDonald and Chen, 1964) have mad e the study of the enzymic hydrolysis of barley and malt proteins a difficult one. In contrast, the proteolytic enzymes of the gastro-intestinal tract of higher animals have been studied in considerable detail and the information available from these studies may provide a useful background for the study of barley and malt proteolytic enzymes.

The results presented here indicate that the malt extract, prepared as described earlier (see EXPERIMENTAL section), contains enzymes which are able to hydrolyse the peptide linkages of bovine haemoglobin, an azo-derivative of the wheat protein glutenin and of the synthetic compound α - benzoyl - L - arginine - p - nitroanilide (BAPA); the ester linkage of α - benzoyl - L - arginine ethyl ester (BAEE); and the amide linkage of benzoyl - L - arginamide (BAA). However, the same malt extract did not hydrolyse another synthetic substrate, N - acetyl - L - tyrosine ethyl ester (ATEE), specific for chymotrypsin, when assayed according to the method of Schwert and Takenaka (1961). This indicated the inability of the malt enzymes to hydrolyse, under the experimental conditions described, the ester linkage when in the synthetic substrate the basic amino acid side chain was replaced by the aromatic amino acid tyrosine. Since BAPA, BAEE AND BAA are all specific substrates for animal trypsin (Davis and Smith, 1961), which preferably hydrolyses the peptide, ester or amide linkages of carboxyl (COOH) groups of the basic amino acids lysine or arginine attached to other compounds, it may appear at first consideration that extracts of malt contain an enzyme or enzymes which are akin to animal trypsin in their substrate specificity. Indeed such a view has been taken by Enari et al. (1963) who aggested that water extracts of barley and malt contain an enzyme which resembles animal trypsin in its substrate specificity and active centre. Such a claim should, however, be accepted with considerable reserve, as hydrolysis of a substrate, even though of a known structure, is not adequate evidence for the presence of a new enzyme. Furthermore different enzymes may possess homo-synthetic substrate specificity as has been shown in the case of trypsin, ficin, bromelain and papain, which are all able to hydrolyse the synthetic substrate benzoyl - L - arginamide to benzoyl - L - arginine and ammonia (Whitaker, 1961). Nevertheless, the reports of Enari and Associates (Enari et al., 1963; Suolinna et al., 1965) together with the report of Burger (1966) constitute the only findings recently reported in the literature in which a serious attempt has been made to study the proteolytic enzymes of barley and malt. The present results of fractionation of malt proteolytic enzymes are discussed mainly in the light of these findings.

Choice of Synthetic Substrate.

In the fractionation studies reported here, the substrate BAPA was used along with Hb in preference to the other synthetic substrates BAEE and BAA. BAPA was selected because of its use in the enzyme assay of barley and malt proteolytic enzymes by all the previous workers (Enari and Coworkers, 1963; Suolinna and Coworkers, 1965; Burger, 1966). Furthermore the assay conditions have been fully established, the assay method being sensitive and convenient. The yellow colouration obtained on the release of p - nitroaniline by enzymic action (Fig. 2) is easily recognisable. In contrast considerable difficulty was encountered when attempts were made to employ BAA as a substrate. The ammonia liberated could not be estimated by Nesslerization, as the unhydrolysed substrate was precipitated by the Nessler reagent. The Conway technique for determination of the ammonia released was also found unsuitable because of the low enzyme (BAA-ase) activity in the column fractions. The alternative of distilling off the ammonia released in a micro-Kjeldahl assembly in the presence of concentrated sodium hydroxide was not found attractive because of the possiblity of alkaline hydrolysis of the amide bond. However, when this method of assay was employed with the ammonium sulphate fractions (Table XXI), little hydrolysis of the amide bond occurred as the assay blank was not unusually high.

It may be pertinent to consider whether the synthetic substrates BAPA, BAEE and BAA are hydrolysed by one barley or malt proteolytic enzyme having a broad substrate specificity as in the case of animal trypsin, or by three different enzymes each specific for the peptide, ester or the amide linkages. Burger (1966) concluded that BAEE-ase and BAA-ase are different, since upon fractional precipitation with ammonium sulphate, maximum precipitation of BAEE-ase was obtained between 50 to 60% salt saturation and that of BAA-ase between 40 to 50% salt saturation; the ratios of the enzymes activity being constant. This worker further concluded, on the basis of evidence obtained from ion-exchange chromatography on DEAE-cellulose, that BAA-ase and BAPA-ase are discrete enzymes. BAA-ase was eluted, from the ion-exchange column, as a basic protein, while BAPA-ase has been reported to be present in the acid proteins of barley and malt fractionated, on DEAE-cellulose chromatography, by Enari et al. (1963). The relationship of BAEE-ase to BAPA-ase has not been investigated so far. The present evidence does not conclusively support Burger's findings as in the fractionation studies conducted here BAPA-ase was usually employed as a substrate. Only with the ammonium sulphate fractions (Table XXI) an additional substrate BAEE was used. In this case both BAPA-ase and BAA-ase were precipitated maximally at the same salt concentration (40 to 60%). Although further corroboration is required, nevertheless, it would appear from Burger's work that more than one enzyme is involved in the hydrolysis of BAPA, BAEE and BAA.

Since BAPA does not possess free amino or carboxyl groups (see Fig. 2), one would expect this substrate to be hydrolysed by endopeptidases and not by exopeptidases (see classification of proteolytic enzymes earlier). Burger (1966) has, however, claimed, on the basis of the inability of BAPA-ase to reduce the viscosity of gelatin, that BAPA-ase may be a peptidase with little or no proteinase activity. Unfortunately, Burger (1966) did not substantiate his claim by employing as substrates synthetic di- or tripeptides. Although the presence of peptidases, which hydrolyse the synthetic di- and tripeptides, in green malt has been previously reported (Linderstrom-Lang and Sato, 1929), it is, however, difficult to visualize, in view of the structure of BAPA, that this substrate is hydrolysed by an enzyme other than an endopeptidase.

Studies on Hb-ase.

Preliminary experiments conducted to establish optimal assay conditions for the assay of Hb-ase showed that maximum enzyme activity, under the experimental conditions described, was obtained at a pH close to 3.8 (Table XIV and Fig. 5) and at a substrate concentration of 1.0 to 1.5% (Table XVIII and Fig. 9), the higher substrate concentrations being inhibitory to the enzyme. At first consideration, a pH optimum in the vicinity of 3.8 as obtained for Hb-ase in these studies would seem low for an enzyme of plant origin, the optimum pH of papain, the most extensively studied plant enzyme with haemoglobin as substrate being 7.5 (Davis and Smith, 1961). However, after these results were obtained it was noted that McDonald and Chen (1964) also reported a pH optimum of 3.8 for wheat flour proteinase and later Burger (1966) for barley proteinase, haemoglobin being used as substrate in both cases. The evidence obtained here would thus suggest that a major component of barley and malt proteolytic enzymes, probably like other cereal proteolytic enzymes, possesses a pH optimum in the acid range. Supporting this view are the pH optima of the proteolytic enzymes in malt extracts acting on edestin (pH 4.3), egg albumin (pH, 4.6), casein (pH 3.5 - 5.7) reported by Harris (1962) and gelatin (pH, 5.2) reported by Enari and Coworkers (1963).

The Hb-ase appeared to be relatively thermostable when heated for five minutes at various temperatures. Thermolability of the enzyme started at 45°C. At 70°C, the enzyme still possessed some activity (Table XV and Fig. 6). This contrasts with the complete loss of activity of the soluble fractions of proteolytic enzymes of malt at the same temperature within two minutes of heating reported by Kringstad and Kilhovd (1957).

Separation of proteolytic Enzymes of malt by Column Chromatography.

The poor separation of malt proteolytic enzymes by exclusion chromatography on Sephadex G-75 with haemoglobin as substrate (Fig. 11) and on Sephadex G-100 (using the ammonium sulphate precipitated extract) with α - benzoyl - L - arginine - p - nitroanilide as an additional substrate (Fig. 13) suggests that the malt enzymes either possessed similar or nearly similar molecular weights. Alternatively, they may have been present in the freeze-dried extract as an agglomerate in combination with other proteins and possibly other substances (in exclusion chromatography on Sephadex G-75 and G-100, the enzyme peaks always appeared in coincidence with the first protein peak, see Fig. 11 and 13) and were eluted as such. The explantion that the malt enzymes possessed similar molecular weights, though not unreasonable, is difficult to accept in view of the results reported by Enari and Associates (1963) who fractionated ~~the~~ proteolytic enzymes of barley and malt by gel filtration on Sephadex G-100 assaying the proteolytic activity by measuring reduction in the viscosity of gelatin into a number of peaks. Latterly, Burger (1966) also reported the resolution of green malt extract into two regions when chromatographed on Sephadex G-100 using haemoglobin as a substrate. It should, however, be pointed out that the results obtained by these workers may not be directly comparable to those obtained here as both Enari and Coworkers and Burger had used, for exclusion chromatography, extracts which were relatively pure as they were previously chromatographed on ion-exchange (DEAE-cellulose) resin. Furthermore, Enari and Coworkers (1963) used for exclusion chromatography extracts which were obtained from grain treated with acetone. In the present study, the effect of acetone treatment of the grain was investigated on the release of Hb-ase only (Table VII). Whether such a treatment of grain facilitates resolution of proteins on the gel column by separating enzymes from attached proteins and other materials cannot be said with certainty.

When fractionation of malt proteases was attempted by chromatography on DEAE-cellulose using step-wise elution, all the proteolytic activity appeared in the basic or neutral fraction eluted with 0.1M orthophosphate buffer, pH 7.0 and none in the acid fraction eluted with 0.5M orthophosphate buffer, pH 4.0, though this latter fraction contained substances which absorbed at 280 mμ (Fig. 12). These results agree with those of Enari and Mikola (1961) who by using the same technique as employed here, had reported the distribution of 75% of protease activity in the basic protein fraction, 25% in the neutral protein and none at all in the acid fraction. They do not agree however, with the results reported by Enari and Associates (1963) in a later paper. In this later paper they reported one half of the proteolytic activity to be present in the neutral proteins and the other half to be present in the acid proteins; all of the BAPA-ase activity being present in the acid proteins. Although Enari and Associates did not explain the apparent discrepancy in their results, it may well be due to difficulty of precisely separating the proteolytic enzymes into basic, neutral or acid fractions. Nevertheless, the elution pattern obtained here on ion-exchange chromatography did not show any resolution as achieved by Enari and Coworkers though the technique used was essentially the same. No satisfactory explanation can be forwarded for the apparent variation in the present results from those of Enari and Associates except that it may well be due to different material chromatographed here. Quantitative variation, though small, in the basic proteins between different samples of one barley variety and different varieties of barley have been reported by Mikola (1965).

Studies with Inhibitors.

The inhibition of Hb-ase by potassium bromate, the sulphhydryl blocking compounds, phenylmercuryacetate and p-chloromercuriphenylsulphonic acid, and the alkylating agent, N-ethylmaleimide, (see RESULTS section) strongly indicated that a papain-type of enzyme or group of enzymes are involved in the hydrolysis of haemoglobin at pH 3.8. This view was further confirmed by the activation of this enzyme by ethylenediamine tetraacetic acid. That barley and malt contain, like wheat, enzymes in which thiol groups are involved in the active centre is well known. To that extent, these results are in agreement with those previously reported (p. 18). It is difficult to advance a satisfactory explanation for the lack of inhibition of Hb-ase by iodoacetamide. The other alkylating agent N-ethylmaleimide also showed only slight inhibition of Hb-ase and that too at the highest concentration employed (1×10^{-2}M). Lack of inhibition of wheat flour proteolytic enzymes at pH 3.8 by these two compounds has also been reported by McDonald and Chen (1964). N-ethylmaleimide strongly inhibited BAPA-ase at an alkaline pH of 8.6. Presuming that BAPA-ase is an -SH type of enzyme (see later), it would appear that this compound is an effective inhibitor of -SH groups in the alkaline range of pH. The same cannot, however, be said of iodoacetamide, which had little effect on the BAPA-ase activity.

The lack of inhibition of BAPA-ase by potassium bromate and iodoacetamide (small or negligible inhibition only at a concentration of 1 x 10^{-1}M) agrees with the findings of Enari and Coworkers (1963), Suolinna and Coworkers (1965) and Burger (1966). Nevertheless, its strong inhibition by the mercury compound, p-hydroxymercuribenzoate and to a lesser extent by N-ethylmaleimide and its activation, like the Hb-ase, by ethylenediamine-tetracetic acid (see RESULTS section) suggests that BAPA-ase may also be a papain type enzyme. Furthermore the lack of inhibition of BAPA-ase by phenylmethylsulphonylfluoride, a specific trypsin inhibitor which inhibited commercial trypsin under identical assay conditions, further supports this view. Unfortunately this trypsin inhibitor could not be tested on the Hb-ase because of its lack of solubility in buffers of acidic pH. Results of inhibitor studies with BAPA-ase reported by Enari and Associates are highly conflicting, although these were conducted with purer BAPA-ase preparations than that used in the present study. Enari and Coworkers (1963), for example, reported in their first paper that barley and malt BAPA-ase was not inhibited by potassium bromate, iodoacetic acid or ethylenediamine-tetraacetic acid but by a small molecular weight inhibitor present in the water extract of the grain. Removal of this inhibitor by gel filtration on Sephadex G-25 caused a fourfold increase in the activity of BAPA-ase. These results thus implied that BAPA-ase may have an active centre like that of animal trypsin. In a later paper, however, these workers (Suolinna et al., 1965) reported that BAPA-ase was inhibited by -SH reagents particularly by p-chloromercuribenzoic acid and by N-ethylmaleimide, the alkylating agent. These results are thus in close agreement with those obtained in this study. However, Suolinna et al. concluded that the thiol group in BAPA-ase is not present at the active centre but sufficiently close to it to interfere with the formation of enzyme-substrate complex. These workers did not test any specific trypsin inhibitor on their BAPA-ase preparation as had been done in the present study. Evidence similar, to that obtained here has also come from the work of McDonald and Chen (1964) who reported a lack of inhibition of wheat proteolytic enzymes by trypsin inhibitors from soybean, lima bean, egg (ovomucoid) and by p - nitrophenyltrimethylacetate, which inhibits both trypsin and chymotrypsin.

These results, therefore, as far as they go, do not support the claim of Enari et al. (1963) that the barley and malt enzyme which catalysed the hydrolysis of the synthetic substrate α - benzoyl - L - arginine - p - nitroanilide (BAPA) at an alkaline pH (8.6) resembles trypsin in its specificity and active centre. Most likely, BAPA-ase is another of the -SH type cereal proteolytic enzyme and is most probably akin to other proteolytic enzymes of plant origin like papain, ficin and others.

If both BAPA-ase and Hb-ase have a similar active centre as shown by the evidence obtained in this study, it is then pertinent to consider whether BAPA-ase and Hb-ase are one and the same enzyme. The evidence obtained here, though limited, suggests the contrary. The pH optimum of the Hb-ase was obtained in the acidic range (3.8) and that of the BAPA-ase has been reported in the alkaline range (8.6). It is unusual for an enzyme to have a pH optimum in the acid as well as in the alkaline range. Furthermore, the partial separation of Hb-ase and BAPA-ase in exclusion chromatography on Sephadex G-100 (Fig. 13) provides additional evidence to support this view. A consistant decrease in the Hb-ase activity of green malt extract on purification of BAPA-ase reported by Burger (1966) suggests a similar conclusion.

(C) Development of Proteases in Germinating Barley.

Inspite of a wide variation in their pH optimum, the formation of the Hb-ase and the BAPA-ase in barley germinating at 13 - 15°C was parallel (Fig. 14). Similarly the increase in the enzyme activity on germination and its maximum development on the fifth day of germination in both the enzymes followed a close pattern. As far as the development of the total proteolytic activity is concerned (measured here by the Hb-ase at pH 3.8), these results are in close agreement with those reported by others. For example, Urion (1950), Kringstad and Kilhovd (1957) and more recently Enari et al. (1963) have all reported a maximum development of proteolytic activity in germinating barley on the fifth day. The close pattern of development of both the enzymes appeared to suggest that endopeptidases (BAPA-ase being an endopeptidase according to Enari et al. 1963) probably develop concomitantly in germinating grain. Waldschmidt-Leitz and Purr (1931) have reported that the development of endopeptidases precedes the development of exopeptidases and amylases. This order of development of proteolytic enzymes in germinating grain may be expected as reserve proteins need to be hydrolysed first by proteases to smaller fragments before peptidases can come into play and hydrolyse these fragments to amino acids.

A decrease in the soluble protein content of the grain after steeping till the second day of germination (Fig. 14), when there was little corresponding enzyme activity and hence no hydrolysis of the reserve proteins, appeared to indicate utilization of the soluble proteins in 'situ' by the germinating grain. Thereafter with the development of the enzyme activity, a concomitant hydrolysis of the reserve proteins occurred. A later decrease in the soluble protein content (after the fifth day of germination) suggested that at this stage the initial essentially degradative phase was replaced by one in which increasing synthesis of the cytoplasmic proteins occurred. Such a conclusion has also been reached by Kolbach (1955) who suggested that during germination hydrolysis and synthesis proceed simultaneously, hydrolysis predominating until the fifth day (as observed in the present study, see Fig. 14) when equilibrium between the two processes is established. Subsequently, the imbalance between hydrolysis and synthesis is less marked because of the increasing contribution of the latter.

SUMMARY AND CONCLUSIONS

(A) Autolysis of Barley and Malt.

1. Proteolytic activity of six malting barley varieties, as measured by the release of the Water-Soluble, Formol and Non-Protein nitrogen from grain, autolysed at 40°C with distilled water, showed no significant varietal differences. The varieties, therefore, could not be selected on the basis of proteolytic activity.
2. The production of the Water-Soluble, Formol and Non-Protein nitrogen in barley and malt was inhibited by phenylmercuryacetate, iodoacetic acid and activated by thioglycollic acid. The activation or inhibition of the nitrogen fractions was small. Nevertheless, it suggests that autolysates of barley and malt contain papain-type enzymes, though other different types of proteolytic enzymes may also be present.
3. The oxidizing agent potassium bromate showed no effect on the proteolytic activity of barley or malt autolysates.

(B) Fractionation of Proteolytic Enzymes of Malt.

1. A freeze-dried extract of malt hydrolysed commercial bovine haemoglobin (Hb), an azo-derivative of the wheat protein glutenin. It also hydrolysed three synthetic substrates, α - benzoyl - L - arginine - p - nitroanilide (BAPA), α - benzoyl - L - arginine ethyl ester (BAEE) and benzoyl - L - arginamide (BAA), all specific for animal trypsin. The results indicated the presence in malt of proteolytic enzymes able to hydrolyse peptide, ester and amide bonds of these synthetic substrates.

2. An optimum hydrolysis of haemoglobin occurred in the vicinity of pH 3.8 and at a substrate concentration of 1.0 to 1.5%.
3. The enzyme (s) which hydrolysed haemoglobin (Hb-ase) at pH 3.8 was thermostable up to 40°C, when heated for five minutes.
4. Fractionation of the proteolytic enzymes of malt was attempted by ammonium sulphate fractionation, exclusion chromatography on Sephadex G-75 or G-100 and by DEAE-cellulose chromatography. A partial separation of Hb-ase and BAPA-ase was obtained by exclusion chromatography on Sephadex G-100.
5. The Hb-ase was inhibited by potassium bromate, phenylmercury acetate, p - chloromercuriphenylsulphonic acid, N-ethylmaleimide (slightly) and activated by ethylenediaminetetraacetic acid, suggesting that -SH groups were involved in the enzyme activity.
6. The BAPA-ase was strongly inhibited by p - hydroxychloromercuribenzoate and to a lesser extent by N - ethylmaleimide and like Hb-ase was activated by ethylenediaminetetraacetic acid. It was not inhibited by potassium bromate iodoacetamide (negligible inhibition) or by phenylmethylsulphonylfluoride, a specific trypsin inhibitor, which inhibited commercial trypsin under identical assay conditions.
7. The evidence obtained with the use of inhibitors suggested that BAPA-ase was not similar in is mechanism of action to trypsin but probably to another of the -SH type plant proteolytic enzyme.
8. The evidence, though limited, indicated that Hb-ase and BAPA-ase are separate enzymes.

(C) Development of Proteases in Germinating Barley.

1. The formation of Hb-ase and BAPA-ase was parallel in barley germinating at 13 to 15°C.

2. Maximum development of both the enzyme reached on the fifth day of germination, after which the enzyme activities were constant.

3. The increase in activity of both the enzymes, on germination, was threefold.

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